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ESTUDO DO PERFIL DA RESPOSTA IMUNE POR ANTICORPOS EM DIFERENTES FORMAS CLÍNICAS DE LEPTOSPIROSE A PARTIR DE UM MICROARRANJO DE PROTEÍNA DO GENOMA CODIFICANTE COMPLETO DE *LEPTOSPIRA INTERROGANS* SOROVAR COPENHAGENI

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Orientador: Prof. Dr. Marco Alberto Medeiros

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“- Aprendi - respondeu Tistu - que a medicina não pode quase nada contra um coração muito triste. Aprendi que para a gente sarar é preciso ter vontade de viver. Doutor, será que não existem pílulas de esperança?
- Você aprendeu sozinho a primeira coisa que um médico deve saber.
- E qual é a segunda, doutor?
- É que para cuidar direito dos homens, é preciso amá-los bastante.”

Maurice Druon

Ministério da Saúde

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

Carolina Lessa Aquino

A leptospirose é uma doença zoonótica causada por bactérias do gênero *Leptospira*. A escassez de informação sobre a resposta imune associada à infecção dificulta não somente o desenvolvimento de um teste diagnóstico rápido e diferencial para leptospirose, mas também de uma vacina eficaz. O objetivo desse trabalho foi utilizar uma abordagem proteômica de alto desempenho para aprimorar o entendimento da resposta imune humoral associada à infecção natural por leptospira. Para isso, a resposta por anticorpos IgM e IgG de pacientes com leptospirose branda e grave, bem como de indivíduos saudáveis, foi avaliada frente a um microarranjo de todas as proteínas preditas para o genoma de *L. interrogans* sorovar Copenhageni. Uma análise de enriquecimento de categorias de proteínas foi realizada, identificando-se características potencialmente associadas à sororreatividade, tais como presença de peptídeo sinal, domínio transmembrana e localização subcelular em membranas. Além dessa análise, os pacientes foram comparados aos indivíduos saudáveis com o intuito de identificar antígenos com potencial diagnóstico e/ou vacinal para leptospirose. Ao todo, 36 antígenos foram capazes de discriminar entre casos agudos ou convalescentes de leptospirose e indivíduos saudáveis, sendo, portanto, candidatos potenciais para um novo teste diagnóstico e/ou vacina para leptospirose. Adicionalmente, foi observado que pacientes com leptospirose grave apresentaram aumento significativo nos níveis de IgG da fase aguda para fase convalescente, característica de uma infecção primária por leptospira, enquanto em pacientes com manifestações brandas, os níveis de IgG se mantiveram estáveis com a progressão da doença, sugerindo uma resposta anamnéstica nesses pacientes. Em conjunto, os resultados obtidos demonstraram que pacientes infectados naturalmente por leptospira desenvolvem uma resposta imune humoral intensa que, potencialmente, pode conferir proteção contra o desenvolvimento de leptospirose clínica e de suas manifestações graves.

INSTITUTO OSWALDO CRUZ

STUDY OF THE ANTIBODY RESPONSE IN LEPTOSPIROSIS PATIENTS WITH DIFFERENT CLINICAL MANIFESTATIONS USING A PROTEIN MICROARRAY CHIP COMPRISING THE *LEPTOSPIRA INTERROGANS* SEROVAR COPENHAGENI COMPLETE CODING GENOME

ABSTRACT

PhD THESIS IN CELLULAR AND MOLECULAR BIOLOGY

Carolina Lessa Aquino

Leptospirosis is a zoonotic disease of global importance, caused by bacteria of the genus *Leptospira*. The poor understanding of the immune response during *Leptospira* infection hinders not only the development of rapid and reliable diagnostic tests but also of new efficacious vaccines. The aim of the present study was to apply high-throughput proteomic tools to improve the understanding of the immune response during naturally acquired leptospirosis. For that, we used a protein microarray chip to identify the IgM and IgG antibody profiles of patients with severe and mild leptospirosis, as well as healthy individuals, against the complete *Leptospira interrogans* serovar Copenhageni predicted ORFeome. An enrichment analysis identified proteomic features associated with antibody recognition, for instance, positive prediction for signal peptide, transmembrane domain and sub-cellular localization as outermembrane. Comparing patients with controls, we identified a set of 36 antigens specific to patients, which are potential serodiagnostic and/or subunit vaccine candidates. Additionally, we observed that in the severe group, IgG responses increase over time, while it remains relatively the same in the mild patient group, which led us to hypothesize that patients with mild symptoms were having an anamnestic response to the pathogen, while the profile of patients with severe outcomes was representative of a first exposure. Altogether, these findings show that leptospirosis patients develop a robust antibody response during infection, which might be protective against clinical leptospirosis and severe outcomes.

ÍNDICE

LISTA DE SIGLAS E ABREVIATURAS

BA	Bahia
CD8	do inglês, *Cluster of differentiation 8*
CDS	do inglês, *Coding sequence*
DNA	do inglês, *Deoxyribonucleic acid*
DOI	do inglês, *Digital Identifier Object*
ELISA	do inglês, *Enzyme-linked immunosorbent assay*
icMS	do inglês, *Immunocapture mass spectrometry*
IgM	Imunoglobulina M
IgG	Imunoglobulina G
IL-2	Interleucina-2
INF- γ	Interferon- γ
JCVI	do inglês, *John Craig Venter Institute*
LERG	do inglês, *Leptospirosis burden epidemiology reference group*
Lig	do inglês, *Leptospiral immunoglobulin-like*
LPS	Lipopolissacarídeo
LC-MS	do inglês, *Liquid chromatography mass spectrometry*
TOF	do inglês, *Time of flight*
MALDI	do inglês, *Matrix-assisted laser desorption ionization*
MAT	do inglês, *Microagglutination test*
NCBI	do inglês, *National Center for Biotechnology Information*
NLR	do inglês, *Nod-like receptor*
PBMC	do inglês, *Peripheral blood mononuclear cells*
PCR	do inglês, *Polymerase chain reaction*
PAGE	do inglês, *Polyacrylamide gel electrophoresis*
PVDF	do inglês, *Polyvinylidene fluoride*
RNAm	do inglês, *Messenger ribonucleic acid*
SDS	do inglês, *Sodium dodecyl sulfate*
SERPA	do inglês, *Serological proteome analysis*
SEREX	do inglês, *Serological analysis of recombinant expression libraries*
SPH	Síndrome pulmonar hemorrágica
Th2	do inglês, *T helper 2*

TNF-α	do inglês, *Tumor necrosis factor-α*
TLR	do inglês, *Toll-like receptor*

1 INTRODUÇÃO

1.1 Classificação genética e sorológica

O gênero Leptospira pertence à família Leptospiraceae, filo Spirochaetes. A atual classificação das espécies é baseada na relação genética e indica a existência de pelo menos 20 espécies diferentes, distribuídas filogeneticamente em 3 grupos: 9 espécies patogênicas, 5 espécies intermediárias e 6 espécies saprofíticas. As espécies são, ainda, categorizadas em mais de 200 sorovares, de acordo com a presença ou ausência de aglutinação frente aos soros heterólogos. O sorovares antigenicamente relacionados são classificados em sorogrupos (Ko et al., 2009; Evangelista and Coburn, 2010; Levett, 2015).

A classificação sorológica das espécies leva em consideração a heterogeneidade estrutural do lipopolissacarídeo (LPS). As diferenças estruturais no lipídeo A do LPS determinam a diversidade antigênica entre os inúmeros sorovares. Tal classificação não apresenta relação taxonômica, de modo que um sorovar pode estar relacionado a diferentes espécies. No entanto, a classificação sorológica é extremamente útil no diagnóstico laboratorial inicial da leptospirose e em estudos epidemiológicos (Evangelista and Coburn, 2010; Fraga et al., 2011; Levett, 2015).

Entre as espécies patogênicas que constituem os principais agentes da leptospirose humana e animal, estão: *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri* and *L. alexanderi* (Evangelista and Coburn, 2010). A espécie empregada no presente estudo foi *L. interrogans* sorovar Copenhageni, pertencente ao sorogrupo Icterohaemorrhagiae, que é o principal agente causador da leptospirose urbana no Brasil (Ko et al., 1999; de Faria et al., 2008).

1.2 Biologia do micro-organismo

As leptospiras são bactérias alongadas, delgadas, em formato espiral e com alta motilidade. Espécies saprofíticas, como *Leptospira biflexa*, são organismos de vida livre encontrados no solo e na água e, diferentemente das espécies patogênicas, não infectam hospedeiros animais (Ko et al., 2009; Fraga et al., 2011). Já as espécies patogênicas são capazes de infectar uma grande variedade de hospedeiros animais e de sobreviver tanto em ambiente marinho como em solo e ambientes de água doce. Apesar dessa diversidade, características como tamanho, forma, motilidade e ultraestrutura são semelhantes em todas as espécies (Ko et al., 2009; Evangelista and Coburn, 2010; Fraga et al., 2011; Cameron, 2015).

As leptospiras apresentam diâmetro aproximado de 0,1 µm e comprimento variando de 6 a 20 µm (Ko et al., 2009; Evangelista and Coburn, 2010; Fraga et al., 2011; Cameron, 2015). O envelope celular mostra características de bactérias Gram-negativas, como a presença de membrana externa, espaço periplasmático e LPS, e também de bactérias Gram-positivas, como a associação de uma espessa camada de peptidoglicano à membrana interna da bactéria. Assim como outras espiroquetas, as leptospiras possuem 2 endoflagelos ancorados no espaço periplasmático, que são responsáveis por sua intensa motilidade, incluindo três tipos de movimentos distintos: progressivo, circular e ao redor do eixo central (Bharti et al., 2003; Fraga et al., 2011; Cameron, 2015). A sua forma característica de gancho ou ponto de interrogação a difere das demais espiroquetas (Ko et al., 2009; Evangelista and Coburn, 2010; Fraga et al., 2011; Cameron, 2015).

As leptospiras são micro-organismos aeróbios estritos, com crescimento lento e ótimo entre 28-30°C, em pH 7,2 a 7,6. Sua maior fonte de energia é proveniente da beta-oxidação de ácidos graxos de cadeia longa. A não utilização da glicose como fonte primária de carbono se dá, não pela ausência de vias de degradação, mas pelo seu limitado sistema de transporte de glicose (Nascimento et al., 2004; Cameron, 2015). Outros compostos requeridos para

seu crescimento incluem nitrogênio, fosfato, cálcio, magnésio, ferro, cobre, manganês e sulfato (Cameron, 2015).

A organização genômica das leptospiras consiste em 2 cromossomos circulares, um maior e outro menor, totalizando de 3,9 a 4,6 Mb, dependendo da espécie. Trata-se de um genoma relativamente extenso comparado a outras espiroquetas, tais como *Treponema spp*. e *Borrelia spp*., cujos genomas possuem aproximadamente 1,1 Mb e 1,5 Mb, respectivamente (Bharti et al., 2003; Cameron, 2015). Essa notável diferença pode estar relacionada à habilidade das leptospiras em viverem em condições muito diversas, como dentro do hospedeiro animal ou livre no ambiente (Bharti et al., 2003). No caso da cepa empregada nesse estudo, *Leptospira interrogans* sorovar Copenhageni, linhagem Fiocruz L1-130, o genoma foi sequenciado e anotado pelo nosso grupo (Nascimento et al., 2004); evidenciou-se um cromossomo maior, com 4.227.185 pb e 3.399 sequências codificantes (CDS) e o cromossomo menor, com 350.181 pb e 268 CDS.

1.3 Aspectos de patogenia

A bactéria pode penetrar no hospedeiro através das membranas mucosas dos olhos, nariz e garganta ou da pele lesionada, estabelecendo rapidamente uma infecção sistêmica por disseminação hematogênica (Bharti et al., 2003; Ko et al., 2009; Evangelista and Coburn, 2010). A disseminação da infecção no hospedeiro envolve a quebra de muitas barreiras, incluindo matriz extracelular, membranas basais e camadas celulares, mas os mecanismos moleculares envolvidos nesses eventos permanecem incompreendidos (Murray, 2015).

Há evidências de que a bactéria atravessa camadas celulares, passando não somente entre as células, como também através delas, seja internalizada em vesículas ou livre no citoplasma (Marshall, 1976; Thomas and Higbie, 1990; Barocchi et al., 2002). Associado a isso, o genoma desses organismos codifica uma grande variedade de hemolisinas e proteases (Ko et al., 2009;

Palaniappan et al., 2007). As proteases bacterianas parecem atuar na disseminação através da matriz extracelular, enquanto as hemolisinas possuem atividade lítica não só em eritrócitos, mas também em outros tipos celulares (Evangelista and Coburn, 2010; Vieira et al., 2013). Acredita-se, ainda, que a alta motilidade das leptospiras também desempenhe papel fundamental na penetração e disseminação bacterianas (Murray, 2015).

Inúmeros determinantes de virulência, como proteínas de superfície e de secreção e LPS, já foram caracterizados e apresentam potencial contribuição para a patogênese da infecção e doença (Bharti et al., 2003; Palaniappan et al., 2007). Mutantes com LPS modificado apresentaram acentuada atenuação no modelo de infecção aguda (Murray et al., 2010; Srikram et al., 2011). Porém, sabe-se que a toxicidade do LPS das leptospiras é inferior à de outras bactérias Gram negativas (Evangelista and Coburn, 2010; Fraga et al., 2011). Os demais fatores de virulência identificados até o momento, com demonstração experimental, são primariamente proteínas de superfície relacionadas à interação da bactéria com os tecidos do hospedeiro (Ko et al., 2009; Evangelista and Coburn, 2010). De fato, espécies patogênicas expressam um grande número de proteínas que são pelo menos parcialmente expostas na superfície e que reconhecem moléculas da matriz extracelular, entre elas: LigA, LigB, LipL32, LenA e LenB (Murray, 2015).

As proteínas Lig contêm 12 a 13 domínios imunoglobulina-símile repetidos *in tandem* (Matsunaga et al., 2003), cujos genes possuem alto grau de conservação nas diferentes espécies. Evidências experimentais, tais como a regulação gênica positiva quando a bactéria é cultivada em condições fisiológicas de osmolaridade (Malmström et al., 2009) e a perda de expressão das proteínas, com simultânea atenuação bacteriana, em cultivos *in vitro* prolongados (Matsunaga et al., 2003), reforçam sua potencial ação como determinante da virulência durante a infecção natural. A proteína LipL32 é a mais abundante na superfície da célula bacteriana, correspondendo a 75% do conjunto de proteínas de superfície do patógeno e sendo, também, altamente conservada nas espécies patogênicas (Haake et al., 2000; Ko et al., 2009;

Malmström et al., 2009 Evangelista and Coburn, 2010). É importante ressaltar, porém, que as leptospiras patogênicas apresentam extensa redundância gênica e funcional, de modo que mutantes deficientes em tais proteínas não perderam a virulência, possivelmente por tais fatores terem sido compensados por outras proteínas de função relacionada (Adler et al., 2011; Murray, 2015).

1.4 Imunidade do hospedeiro humano

O sistema imune inato constitui a primeira linha de defesa do hospedeiro, desempenhando papel crucial no reconhecimento e eliminação das leptospiras. Duas famílias principais de receptores celulares desempenham papel decisivo no reconhecimento do patógeno pelo sistema imune inato hospedeiro: os receptores semelhantes ao Toll (TLR) e os receptores semelhantes ao Nod (NLR) (Evangelista and Coburn, 2010; Fraga et al., 2011). A maior parte dos estudos em padrão de reconhecimento das leptospiras é focado nos TLRs.

Embora o LPS deste patógeno seja menos tóxico do que de outras bactérias Gram-negativas, ele é capaz de induzir uma forte resposta imune por anticorpos durante a infecção (Chapman et al., 1988). Em um primeiro momento, ele é reconhecido primariamente por TLR2 em humanos, ao invés do TLR4 (Werts et al., 2001). Esse reconhecimento diferenciado é atribuído à composição incomum do lipídeo A e pode ser uma estratégia das espécies patogênicas para evitar a ativação adequada de células do sistema imune, contribuindo para o estabelecimento da infecção em humanos (Palaniappan et al., 2007; Fraga et al., 2011).

Já no contexto da imunidade adquirida, os anticorpos desempenham papel fundamental na imunidade protetora contra infecção letal em muitos hospedeiros mamíferos, incluindo o homem (Adler and Faine, 1976, 1977). Sua produção é importante na fagocitose da bactéria por neutrófilos e macrófagos e inúmeros estudos demonstraram que a fagocitose associada à queda da viabilidade bacteriana é dependente da presença de anticorpos opsonizantes

específicos (Cinco et al., 1981; Banfi et al., 1982; Tu et al., 1982). Isso sugere que o envelope externo das leptospiras possui um componente antifagocítico, possivelmente associado à indução de apoptose em macrófoagos (Ko et al., 2009; Fraga et al., 2011) e/ou à inibição da fusão entre o fagossomo e o lisossomo, com a consequente sobrevivência do micro-organismo (Toma et al., 2011).

Os anticorpos podem, ainda, aglutinar bactérias e ativar a via clássica do sistema complemento. A ativação do sistema complemento é um dos mecanismos efetores mais importantes durante as primeiras horas de infecção e as opsoninas geradas após ativação desse sistema podem também intensificar o processo de fagocitose pelas células do sistema imune inato (Fraga et al., 2011).

Um importante antígeno para o desenvolvimento de imunidade protetora por anticorpos é o LPS. A imunização de *hamsters* com LPS conferiu imunidade protetora em desafio homólogo (Jost et al., 1989) e a transferência passiva de anticorpos anti-LPS também se mostrou protetora em camundongos, porcos da índia, macacos e cães em desafio letal (Jost et al., 1986; Schoone et al., 1989; Challa et al., 2011). Esses anticorpos aparecem no começo da infecção e são sorovar específicos, fornecendo proteção heteróloga limitada (Chapman et al., 1991). Anticorpos contra proteínas da bactéria apresentam mais chances de fornecer proteção cruzada (Sonrier et al., 2000; Srikram et al., 2011).

Apesar do papel crítico das respostas Th2 e de células B na imunidade humoral protetora, linfócitos T também atuam na imunidade do hospedeiro contra a infecção por leptospira. Linfócitos B parecem ser os responsáveis pela produção de interferon- γ (IFN-γ) e eliminação de bactérias no fígado, enquanto linfócitos T possuem função similar nos rins (Chassin et al., 2009). Humanos expostos ao patógeno apresentam uma resposta proliferativa de PBMCs, particularmente células T γδ (Klimpel et al., 2003; Barry et al., 2006;), com expressão de citocinas inflamatórias como IFN-γ, fator de necrose tumoral- α (TNF-α) e receptor para interleucina-2 (IL-2) (de Fost et al., 2003).

Apesar de as leptospiras serem patógenos extracelulares, são micro-organismos com grande capacidade invasiva e de adesão, capazes de penetrar eficientemente na célula hospedeira. Além disso, o escape do fagolisossomo para o citosol de macrófagos humanos é um comportamento típico de patógenos intracelulares (Ko et al., 2009; Fraga et al., 2011). Como consequência, peptídeos bacterianos podem ser apresentados via MHC de classe I para linfócitos T CD8. De fato, linfócitos T CD8 específicos para peptídeos derivados de LigA foram identificados em humanos. No entanto, o papel da imunidade celular na leptospirose permanece pouco compreendido (Palaniappan et al., 2007; Fraga et al., 2011).

1.5 Manifestações clínicas

A infecção por leptospira pode produzir um largo espectro de manifestações clínicas, desde uma doença febril aguda auto-limitada à doença de Weil, a forma grave clássica da leptospirose. Os fatores relacionados ao desenvolvimento da forma grave da doença ainda permanecem pouco compreendidos, mas sabe-se que tanto aspectos relacionados ao micro-organismo – como a espécie, sorovar envolvido e carga infectiva – como aspectos relacionados ao hospedeiro – como idade, estado de saúde e situação imunológica – são importantes (Bharti et al., 2003; Fraga et al., 2011; Picardeau, 2013). O período de incubação desde a exposição ao patógeno até o aparecimento dos sintomas é em média de 7 a 12 dias, podendo variar desde 3 até 30 dias (Morgan et al., 2002; Haake and Levett, 2015). Em sobreviventes, a infecção pode persistir durante meses (Bharti et al., 2003; Fraga et al., 2011; Picardeau, 2013).

A leptospirose costuma ter uma apresentação bifásica, caracterizada por uma fase aguda septicêmica, que dura aproximadamente uma semana, seguida de uma fase imune, que é caracterizada pela produção de anticorpos e desaparecimento dos sintomas (Bharti et al., 2003; Palaniappan et al., 2007; Fraga et al., 2011; Picardeau, 2013). Os sintomas incluem dor de cabeça,

calafrios, náusea e vômitos, mialgia e, menos comumente, “rash” cutâneo. Estes sinais e sintomas são inespecíficos, sendo típicos também de outras doenças febris agudas, como dengue, gripe e malária. A Síndrome de Weil é caracterizada por complicações sistêmicas, com disfunção simultânea de múltiplos órgãos, como fígado, rins, pulmão e cérebro, levando à icterícia, meningite, hemorragias, falências hepática e renal e colapso cardiovascular (Evangelista and Coburn, 2010; Haake and Levett, 2015). A Síndrome Pulmonar Hemorrágica (SPH), que foi associada à leptospirose em um surto na Nicarágua em 1995, vem sendo, desde então, descrita como complicação grave da leptospirose e importante causa de febre hemorrágica em várias regiões do mundo (Marotto et al., 1999; Trevejo et al., 1998; Gouveia et al., 2008; Helmerhorst et al., 2012;). É tipicamente confundida, porém, com uma pneumonia viral (Sehgal et al., 1995; Trevejo et al., 1998). Tais complicações estão, em sua maioria, associadas à localização da bactéria nos tecidos durante a fase imune, ocorrendo, geralmente, durante a segunda semana da infecção.

Alguns indicadores clínicos estão relacionados ao desenvolvimento de formas graves da doença. Estado mental alterado, idade avançada, oligúria e insuficiência respiratória são fatores comumente relacionados a casos fatais (Dupont et al., 1997; Ko et al., 1999; Lopes et al., 2004). Porém, não há, até o momento, marcadores clínicos, moleculares ou sorológicos que possam indicar, no início do curso da doença, se o paciente irá evoluir para desfechos graves. Em geral, o paciente com leptospirose que recebe suporte e tratamento apropriados como, por exemplo, hidratação intravenosa, suplementação de potássio e coquetel de antibióticos adequado, se recupera completamente do quadro infeccioso (Spichler et al., 2011). Em pacientes com SPH, no entanto, a letalidade pode chegar a 50% (Bharti et al., 2003; Helmerhorst et al., 2012).

1.6 Epidemiologia e impacto na saúde pública

Leptospiras patogênicas são amplamente encontradas na natureza, refletindo sua manutenção nos rins de inúmeros animais reservatórios, tanto domésticos quanto selvagens (Haake and Levett, 2015). Quase todos os mamíferos podem ser portadores da bactéria, carreando-a nos túbulos renais proximais (Bharti et al., 2003) (Haake and Levett, 2015). Seu ciclo de vida envolve a eliminação, através da urina dos animais reservatórios, para o meio ambiente, persistência no ambiente até aquisição por um novo hospedeiro e disseminação hematogênica nesse novo hospedeiro até a colonização dos túbulos renais proximais. Nos reservatórios, a bactéria pode permanecer durante anos sem causar danos significativos (Haake and Levett, 2015). Essa habilidade de estabelecer o estado persistente de portador nos animais reservatórios é o componente essencial no ciclo de vida das leptospiras patogênicas.

Os animais reservatórios são comumente chamados de hospedeiros de manutenção, pois são importantes na manutenção da transmissão contínua da bactéria (Ko et al., 2009). Em alguns casos, eles podem atuar como hospedeiros de manutenção para determinados sorovares e como hospedeiros acidentais para outros sorovares, podendo, nessa última condição, evoluir para o quadro agudo da doença. De fato, alguns sorovares de leptospira demonstram preferências específicas por determinados hospedeiros e, em geral, os diferentes sorovares não causam doença nos reservatórios aos quais estão altamente adaptados. É o caso, por exemplo, das cepas do sorogrupo Icterohaemorragiae, que colonizam preferencialmente ratos (*Rattus norvegicus*) (Bharti et al., 2003; Ko et al., 2009).

Os seres humanos, por sua vez, são considerados hospedeiros acidentais, desenvolvendo sinais e sintomas de leptospirose aguda e, algumas vezes, formas fatais da doença (Fraga et al., 2011). A eliminação da bactéria na urina de humanos infectados é transiente, de modo que a transmissão homem-homem da doença é extremamente rara. Já foram relatadas, porém, as

transmissões por via sexual (Harrison and Fitzgerald, 1988) e transplacentária (CHUNG et al., 1963; Coghlan and Bain, 1969; Faine et al., 1984).

Os ratos são considerados o principal reservatório no ciclo de transmissão da leptospirose humana, excretando grandes quantidades da bactéria até meses após a infecção inicial (Palaniappan et al., 2007). Neles, as leptospiras podem causar infecção sistêmica, sendo, em seguida, eliminadas de todos os órgãos, com exceção dos túbulos renais, um sítio imunoprivilegiado; esta característica contribui para o alto grau de persistência do patógeno (Ko et al., 2009).

A exposição humana ao patógeno pode ocorrer através do contato direto com a urina do animal reservatório ou do contato indireto através de água e solo contaminados (Bharti et al., 2003; Ko et al., 2009). O contato indireto é importante para trabalhadores da rede de esgoto, mineradores, limpadores de caixas-d'água e praticante de esportes aquáticos, por exemplo. Já o contato direto se destaca como responsável pela maior parte das infecções em fazendeiros, veterinários, trabalhadores de controle de roedores, entre outros. A transmissão direta está associada, portanto, ao risco ocupacional, enquanto a transmissão indireta pode estar relacionada a riscos ocupacionais ou recreativos (WHO, 2010; Haake and Levett, 2015).

A incidência de leptospirose é significativamente maior em países com clima tropical em comparação com países de clima temperado, já que a bactéria sobrevive por períodos mais longos em ambientes com temperatura e umidade mais elevadas. A doença é sazonal, com pico de incidência ocorrendo no verão ou no outono, nas regiões temperadas – onde a temperatura é um fator limitante para o patógeno – e durante a estação das chuvas, nas regiões tropicais.

Três padrões epidemiológicos distintos foram definidos para leptospirose (Haake and Levett, 2015). O primeiro ocorre nos países de clima temperado e envolve poucos sorovares de leptospira. A infecção humana quase que invariavelmente ocorre pelo contato direto com animais infectados e, nesse

caso, o controle por imunização dos animais e/ou humanos seria potencialmente possível.

O segundo perfil epidemiológico ocorre em áreas tropicais, com a circulação simultânea de inúmeros sorovares e a infecção humana e animal relacionadas a um maior número de animais reservatórios, incluindo roedores, gado e cães. A exposição humana não é limitada por atividades ocupacionais, mas sim, resultante da contaminação ambiental, particularmente durante a temporada de chuvas. Nesse cenário, medidas de controle da população de roedores, drenagem das áreas de inundação e higiene ocupacional são intervenções necessárias para prevenção da leptospirose humana. Essa categoria inclui, ainda, os surtos causados por desastres naturais, como enchentes e furacões.

Na terceira configuração epidemiológica, a doença é causada principalmente por roedores no ambiente urbano. Embora esse perfil seja de menor importância na maior parte do mundo, é potencialmente importante quando a infraestutura urbana é precária. Por esse motivo, tal tipo de infecção raramente é vista em países desenvolvidos, sendo mais comum em áreas onde há altas concentrações de favelas em países em desenvolvimento, como o Brasil (Ko et al., 1999).

A leptospirose é uma doença negligenciada (Hotez et al., 2007), tendo maior impacto em populações carentes de países em desenvolvimento. É, tradicionalmente, a principal causa de mortalidade e morbidade entre agricultores rurais de subsistência (McBride et al., 2005; WHO, 2010) e vem se constituindo como um problema crescente de saúde urbana. Atualmente, 1 bilhão de pessoas residem em favelas (Nations, 2003), marginalizadas da sociedade e sem acesso a serviços básicos de saúde e saneamento. Tais condições facilitam a transmissão da leptospirose a partir dos ratos (Nations, 2003). Estima-se que a população residente em favelas duplique nos próximos 25 anos (Nations, 2003), agravando ainda mais esse cenário (WHO, 2010).

Apesar de ser uma doença comum de países em desenvolvimento, a leptospirose é considerada uma doença emergente, que vem se tornando uma

ameaça à saúde pública também em países desenvolvidos. Atividades recreativas, como viagens, esportes aquáticos e natação, tornaram-se as exposições de risco predominantes nesse contexto (Lau et al., 2010; WHO, 2010). No mundo cada vez mais globalizado, a doença vem emergindo como um importante problema de saúde relacionado a viagens. Além disso, o Painel Intergovernamental sobre as Alterações Climáticas sugeriu que, devido às mudanças climáticas ocasionadas como consequência do aquecimento global, muitas regiões estão susceptíveis a enchentes mais fortes e frequentes (WHO, 2010), favorecendo a transmissão da doença.

A leptospirose tem um impacto significativo na saúde pública, particularmente nos países da Ásia, Américas e África (WHO, 2010; Torgerson et al., 2015). Estimativas recentes indicam que há mais de 500.000 casos de leptospirose por ano ao redor do mundo (WHO, 2010). No Brasil, 2.092 casos confirmados foram registrados entre janeiro e meados de setembro de 2016, dos quais 161 foram fatais. O número de casos por região do país seguiu a seguinte distribuição: 293 casos, com 15 óbitos, na região norte; 178 casos, com 25 óbitos, na região nordeste; 640 casos, com 75 óbitos, na região sudeste; 932 casos, com 43 óbitos, na região sul; e 49 casos, com 4 óbitos, na região centro-oeste (Saúde, 2016). No entanto, é importante ressaltar que o número de casos notificados é significativamente subestimado, pois a notificação é primariamente baseada nas formas graves da doença, uma vez que a forma branda é comumente confundida com outras doenças febris agudas, devido não somente à presença de sinais e sintomas inespecíficos, mas também ao fato de poucos laboratórios no mundo estarem capacitados para realizar os testes diagnósticos padronizados (WHO, 2010; Torgerson et al., 2015).

A prioridade para a saúde pública é a prevenção dos desfechos graves de leptospirose, o que pode ser alcançado, em teoria, pela erradicação das fontes de transmissão ambiental, pelo controle de animais reservatórios, por vacinação e pelo diagnóstico precoce. Porém, até o presente momento, não há uma prevenção efetiva para a leptospirose.

1.7 Vacina contra leptospirose

O primeiro estudo de vacina contra leptospirose foi publicado em 1916, no qual a bactéria inativada foi empregada na imunização de cobaias (Ido et al., 1916). Desde então, o uso de bacterinas em humanos, suínos, bovinos e cães permanece o único tipo de vacina licenciado até o presente (Adler, 2015). Preparações do patógeno inativado por calor ou formol são empregadas há anos na vacinação de animais domésticos (Levett, 2001), prevenindo o desenvolvimento da doença no animal, mas sem efeito sobre o estado de carreador renal (Ko et al., 2009; WHO, 2002). Devido a problemas de reatogenicidade a componentes bacterianos e ao meio de cultura usado no cultivo do micro-organismo inativado, seu uso não obteve a mesma aceitação em humanos (Fraga et al., 2011; Adler, 2015).

Em algumas regiões do mundo, porém, como China, Cuba, Japão e França, vacinas humanas foram licenciadas e são administradas atualmente (Fraga et al., 2011). A imunização é geralmente restrita a indivíduos com alto risco ocupacional, viajantes para regiões com transmissão endêmica da doença e/ou em reposta a enchentes e epidemias (Fraga et al., 2011; Adler, 2015). Essas vacinas apresentam sucesso limitado, produzindo imunidade incompleta de curta duração e restrita a sorovares relacionados (Palaniappan et al., 2007; Adler, 2015). Tal restrição faz com que seja necessário bom conhecimento da epidemiologia regional para uma vacinação eficaz (Adler, 2015).

Sabe-se que a imunidade sorovar-específica está relacionada ao LPS, de modo que vacinas baseadas no antígeno do LPS, embora tenham demonstrado clara proteção em modelo animal, induzem apenas proteção sorovar-específica (Sonrier et al., 2000). Extratos proteicos, por sua vez, podem induzir proteção homóloga e heteróloga, o que faz com que as proteínas do patógeno sejam alvos mais apropriados para o desenvolvimento de uma vacina capaz de induzir imunidade cruzada (Palaniappan et al., 2007). Com o advento das técnicas de DNA recombinante e o sequenciamento

genômico de inúmeras espécies, novos esforços vêm sendo empregados na identificação de antígenos protetores e no desenvolvimento de vacinas de subunidade, de composição definida (Adler, 2015).

Muitas proteínas de membrana externa e lipoproteínas foram identificadas, mas o desenvolvimento de vacinas recombinantes foi demonstrado apenas com certas proteínas de membrana externa, como LipL32, OmpL1, LipL41, LigA e LigB (Ko et al., 2009; Palaniappan et al., 2007 Adler, 2015). No entanto, os estudos realizados com proteínas recombinantes são controversos e, algumas vezes, inconclusivos. Em geral, a eficácia de tais candidatos vacinais é relativamente baixa em modelos animais e a necessidade de uma vacina para auxiliar no controle da leptospirose ainda persiste.

1.8 Diagnóstico laboratorial

Atualmente, a principal barreira para o controle dos desfechos graves da leptospirose é a falta de um teste diagnóstico adequado (Palaniappan et al., 2007). A terapia antimicrobiana pode prevenir a progressão da doença e a alta mortalidade, se administrada aos pacientes precocemente (McClain et al., 1984). Embora a doença grave possa ser reconhecida pelas manifestações clássicas da leptospirose, a identificação clínica dos casos é dificultada pela sua apresentação inespecífica nas fases iniciais (Bharti et al., 2003; Haake and Levett, 2015), de modo que o diagnóstico equivocado tem se tornado ainda mais crítico em regiões onde há transmissão endêmica de doenças infecciosas cujos quadros clínicos são similares. A identificação de casos é, portanto, dependente da anamnese do paciente e do alto grau de suspeita clínica pelos médicos, sendo fundamental, para o diagnóstico precoce, um teste laboratorial que possa ser usado nas unidades primárias de saúde.

O diagnóstico laboratorial da leptospirose pode ser alcançado pela detecção direta do micro-organismo ou seus componentes nos fluidos corporais, por isolamento da bactéria em cultura ou pela detecção de

anticorpos específicos. A coleta da amostra e o teste a ser realizado vão depender do tempo e da duração dos sintomas do paciente (Haake and Levett, 2015). Durante a primeira semana após o aparecimento dos sintomas, quando há leptospiremia, a doença pode ser diagnosticada pelo exame direto do sangue, realizado por microscopia de campo escuro ou imunofluorescência. Cem a 1 milhão de bactérias/mL de sangue podem ser observadas durante a fase aguda da doença, porém o limite de detecção do exame direto é aproximadamente 10^4 bactérias/mL de sangue. Apesar de ser um método barato, sendo necessário apenas um microscópio adequado, o risco de resultados falso-positivos devido à presença de debris celulares, fibrinas ou outros artefatos deve ser considerado (Picardeau, 2013). A observação direta apresenta, portanto, baixa sensibilidade e especificidade (Palaniappan et al., 2007).

Ainda na fase aguda, durante a leptospiremia, é possível recuperar a bactéria a partir de amostras de sangue e cultivá-la em meios especializados. Para isso, é preciso obter histórico acurado do paciente, com data precisa do aparecimento dos sintomas, a fim de aumentar a chance de isolamento do patógeno. Isolamento a partir de amostras de urina, por outro lado, deve ser realizado a partir da segunda semana de sintomas, quando a bactéria começa a ser excretada (Bal et al., 1994; Haake and Levett, 2015). Neste caso, porém, o diagnóstico é apenas confirmatório e já não apresenta tanta relevância no tratamento e manejo do paciente.

O diagnóstico indireto pode ser realizado por sorologia, na busca de anticorpos específicos, ou por técnicas moleculares, que detectam a presença do material genético da bactéria. O DNA de leptospira já foi amplificado a partir de soro, urina, humor aquoso e líquido cefalorraquidiano (Haake and Levett, 2015). Muitos métodos de PCR convencional e PCR em tempo real foram desenvolvidos para detecção do micro-organismo a partir de amostras clínicas, com limite de detecção usualmente entre 10 a 100 bactérias/mL de sangue ou urina (Picardeau, 2013). Uma variedade de genes alvo já foi estudada, podendo ser empregados tanto genes comuns quanto genes específicos para

cepas patogênicas (Smythe et al., 2002; Merien et al., 2005; Palaniappan et al., 2005; Ahmed et al., 2009; Stoddard et al., 2009). Um resultado positivo indica a presença do agente infeccioso na amostra e, no caso da técnica quantitativa, indica, ainda, a carga bacteriana do paciente. Entretanto, uma importante limitação dessas técnicas é a inabilidade de identificar o sorovar infectante que, apesar de não ser relevante para o diagnóstico em si, é importante para estudos epidemiológicos (Picardeau, 2013; Haake and Levett, 2015). Além disso, tal abordagem permanece restrita a laboratórios de referência, sendo improvável seu emprego em larga escala nos países em desenvolvimento, apesar da alta sensibilidade e capacidade de fornecer um diagnóstico precoce (Picardeau, 2013).

A maior parte dos casos de leptospirose é diagnosticada por sorologia. Anticorpos são detectados no sangue de indivíduos infectados a partir de 5-7 dias após o aparecimento dos sintomas (Bharti et al., 2003; Fraga et al., 2011). O uso de testes de aglutinação foi descrito pouco depois do primeiro isolamento do micro-organismo e o Teste de Microaglutinção *in vitro* (MAT) ainda permanece o teste sorológico mais empregado atualmente, sendo considerado o teste padrão-ouro para o diagnóstico da leptospirose (Palaniappan et al., 2007; Picardeau, 2013; Haake and Levett, 2015). O MAT consiste na incubação do soro do paciente com um painel de suspensões de leptospiras vivas de diferentes sorovares (Palaniappan et al., 2007; Picardeau, 2013). As misturas soro-antígeno são avaliadas ao microscópio óptico quanto à presença de aglutinação e os títulos dos anticorpos aglutinantes são determinados através de diluições seriadas da amostra de soro (WHO, 2002). O MAT tem a vantagem de ser altamente específico para os sorovares e, por esse motivo, o espectro de antígenos deve incluir sorovares representativos de todos os sorogrupos, além de todos os sorovares circulantes localmente (Turner, 1968; Haake and Levett, 2015). É importante destacar, porém, que a reatividade cruzada entre os diferentes sorogrupos pode ocorrer, especialmente em amostras de fase aguda (Levett, 2003; Smythe et al., 2009; Murray et al., 2011). A principal desvantagem do ensaio é a baixa sensibilidade

nas amostras de fase aguda, tornando necessário o teste de uma segunda amostra de soro, coletada aproximadamente 15 dias após primeira, para um diagnóstico definitivo e confirmatório. Além disso, para realização do teste é necessária uma infra-estrutura laboratorial adequada, principalmente no que diz respeito à manipulação da bateria de Leptospiras patogênicas. A interpretação do resultado, por sua vez, é subjetiva, pois pode apresentar variação operador-operador e, por esse motivo, requer treinamento e pessoas especializadas. Por fim, o crescimento lento do micro-organismo também dificulta o teste, que é demorado principalmente quando o painel de leptospiras é extenso (Turner, 1968; Bharti et al., 2003; WHO, 2010; Picardeau, 2013).

Devido à complexidade do MAT, outros testes sorológicos para detecção de anticorpos na fase inicial da doença foram desenvolvidos, como ensaios imunoenzimáticos do tipo ELISA e testes rápidos. Anticorpos IgM anti-leptospira podem ser detectados 4 a 5 dias após o aparecimento dos sintomas – antes da detecção de IgG e de anticorpos aglutinantes – e persistem por pelo menos 5 meses nos pacientes (Picardeau, 2013). Inúmeros ELISAs para detecção de IgM estão disponíveis no mercado e baseiam-se na detecção de anticorpos contra o extrato total da bactéria. A sensibilidade desses testes se assemelha à obtida pelo MAT. Entretanto, a especificidade pode ser baixa, uma vez que a cepa saprofítica *L. biflexa* compartilha muitos antígenos de superfície com cepas patogênicas (Haake and Levett, 2015). Os antígenos recombinantes também são comumente empregados para o desenvolvimento de ensaios de ELISA, embora não haja, até o presente momento, uma avaliação em larga escala de testes baseados em proteínas recombinantes (Signorini et al., 2013; Haake and Levett, 2015). A especificidade da detecção de anticorpos IgM por ELISA é dependente do antígeno empregado, da presença de anticorpos gerados em infecções prévias e da presença de doenças concomitantes (Bajani et al., 2003; Haake and Levett, 2015).

Entre os modelos em formato de teste rápido estão as fitas indicadoras (“dipstick”) (Smits et al., 2001b; Levett and Branch, 2002), aglutinação em látex (Smits et al., 2000; Smits et al., 2001a) e testes de fluxo lateral (Smits et al.,

2001b; Nabity et al., 2012). Este último pode ser executado nas unidades básicas de saúde com apenas uma gota de sangue, com resultado disponível em 10 minutos. Consiste na migração do complexo antígeno-anticorpo por capilaridade em membranas cobertas com extrato total da bactéria ou proteínas recombinantes, com a captura do anticorpo através de proteína A e/ou anti-IgM conjugada a ouro coloidal e visualização colorimétrica (Nabity et al., 2012; Picardeau, 2013). Tais testes, em geral, apresentam desempenho característico de outros ensaios, sendo pouco sensíveis na fase inicial da doença, de modo que a confirmação do resultado por um teste de referência é recomendada (Goris et al., 2013; Haake and Levett, 2015).

O atual critério para o diagnóstico da leptospirose, definido pelo grupo de referência em epidemiologia e impacto da leptospirose (LERG), é a presença de sinais clínicos compatíveis com leptospirose associado a: (i) um aumento de 4 ou mais vezes no título de anticorpos entre a primeira e a segunda amostras de soro coletadas; ou (ii) um título único de 1:400 em áreas com transmissão endêmica de leptospirose ou 1:100 em áreas sem endemicidade; ou (iii) isolamento da bactéria a partir de amostras clínicas provenientes de sítios geralmente estéreis, como sangue; ou (iv) detecção de material genético do micro-organismo em amostras clínicas através de PCR. Um caso presuntivo de leptospirose seria determinado pela presença de IgM nos ensaios de ELISA ou “dipstick” ou presença de IgM ou IgG em microscopia de imunofluorescência (WHO, 2010).

1.9 Imunoproteômica e suas aplicações

Um dos principais obstáculos no desenvolvimento de vacinas e testes diagnósticos baseados em sorologia é a identificação de antígenos capazes de induzir a produção de anticorpos ou elicitar uma resposta imune protetora. O advento das tecnologias de sequenciamento de nova geração, contudo, possibilitou um avanço significativo nesse sentido, com o uso de ferramentas de bioinformática que se baseiam na sequência de aminoácidos para predição

de características proteicas comumente associadas à antigenicidade ou imunogenicidade, tais como presença de peptídeo sinal e domínio transmembrana. Tal abordagem seleciona um número consideravelmente reduzido de antígenos com potencial vacinal ou diagnóstico para os ensaios biológicos subsequentes, uma vez que apenas um subconjunto das proteínas do microrganismo apresenta essas características (Pizza et al., 2000). Entretanto, os algoritmos utilizados nessas predições tendem a simplificar excessivamente a condição real, de modo que antígenos importantes podem ser incorretamente descartados (Eyles et al., 2007; Trieu et al., 2011).

Dentre as abordagens empíricas comumente realizadas para complementar ou mesmo aprimorar a abordagem computacional, está a imunoproteômica, que é o estudo em larga escala de proteínas envolvidas na resposta imune de um organismo. De modo mais específico, o termo é usado para descrever o estudo proteômico de antígenos relevantes para a ativação da resposta imune adaptativa, particularmente a produção de anticorpos. Entre os métodos mais empregados estão a análise proteômica sorológica (SERPA), a espectrometria de massas por imuno-captura (icMS), a análise sorológica por bibliotecas de expressão recombinantes (SEREX) e os microarranjos de proteínas. Estudos sobre a relação parasito-hospedeiro por imunoproteômica permitem a identificação de antígenos para diagnóstico e desenvolvimento de vacinas (Jungblut, 2001; Kniemeyer et al., 2016).

Em imunoproteômica, o método mais comum para a identificação de antígenos reativos é o SERPA, que consiste na eletroforese bidimensional de proteínas, seguida de *immunoblottin*g. Ele foi inicialmente empregado na caracterização de antígenos associados a tumores (Klade et al., 2001; Kniemeyer et al., 2016). Nele, as proteínas são primeiramente separadas de acordo com seu ponto isoelétrico, por focalização isoelétrica e, posteriormente, por tamanho molecular, por SDS-PAGE (O'Farrell, 1975; Kniemeyer et al., 2016). O gel bidimensional é, então, transferido para uma membrana de nitrocelulose ou PVDF por técnicas comuns de *western blotting*, para subsequente incubação com soro de pacientes. Anticorpos secundários

apropriados (que reconhecem a porção Fc de IgG) são utilizados para detectar e visualizar o complexo antígeno-anticorpo, de acordo com a técnica de revelação de escolha: fluorescência, quimioluminescência, entre outras. As proteínas antigênicas podem ser localizadas em um gel bidimensional "espelho" revelado com Coomassie e então removidas para digestão proteolítica e identificação por espectrometria de massas (MS) através das técnicas de MALDI-TOF/TOF ou LC-MS/MS. Embora o método apresente alta resolução na separação proteica, a baixa solubilidade de determinadas proteínas e a dificuldade em localizar o sinal obtido por *western blotting* em relação à proteína revelada no SDS-PAGE são fatores que limitam a identificação de antígenos. Outra importante limitação da SERPA é que apenas epítopos lineares podem ser detectados, uma vez que os antígenos são desnaturados durante a eletroforese na presença de SDS (Dutoit-Lefèvre et al., 2015; Kniemeyer et al., 2016).

A icMS consiste na imobilização das imunoglobulinas do soro de pacientes em colunas de proteína A ou proteína G, com a subsequente aplicação de lisados celulares ou sobrenadantes de cultura. As proteínas imunogênicas presentes nesses lisados ou sobrenadantes interagem com o anticorpo imobilizado, de modo a ficarem efetivamente enriquecidas na coluna. Os antígenos imunocapturados são, então, eluídos e submetidos à digestão proteolítica, seguida de identificação por MS *in tandem*. A icMS é uma técnica extremamente eficiente, que permite a triagem simultânea de um grande número de soros de pacientes contra antígenos solúveis, incluindo antígenos de baixa massa molecular. A detecção de proteínas com baixa solubilidade, no entanto, é um fator limitante desta técnica (Kniemeyer et al., 2016).

O microarranjo de proteínas, por sua vez, é um método de alto desempenho para o estudo da reação antígeno-anticorpo (Chang, 1983; Kniemeyer et al., 2016). Proteínas recombinantes, ou mesmo amostras proteicas mais complexas submetidas a fracionamento, podem ser imobilizadas em um chip através de inúmeras técnicas de impressão de microarranjos (Robinson et al., 2002; Barbulovic-Nad et al., 2006; Hueber and Robinson,

2006; Robinson, 2006; Kniemeyer et al., 2016). Estes microarranjos de proteínas são incubados sequencialmente com soro e com anticorpos secundários para detecção e visualização dos antígenos reativos. Quando do uso de amostras proteicas complexas, como extratos celulares fracionados, o antígeno reativo deve ser posteriormente identificado por MS. Neste caso, se forem empregadas técnicas de fracionamento resolutivas, a triagem de antígenos pode permitir a análise do proteoma quase completo de um organismo. O alto desempenho e a baixa quantidade de soro necessária ao ensaio permitem a triagem rápida de centenas de amostras, sem necessidade da utilização de *pools* de soros. No entanto, algoritmos poderosos são necessários para interpretar os dados de maneira confiável (Fulton and Twine, 2013; Kniemeyer et al., 2016).

Em geral, as técnicas de imunoproteômica utilizam extratos celulares submetidos ou não a fracionamento e a identificação dos antígenos mais abundantes é feita por sequenciamento de peptídeos por espectrometria de massas. Uma alternativa ao uso de extratos de células do patógeno como fonte de proteínas é a produção de proteínas recombinantes, baseada na predição de sequências codificantes a partir do genoma do micro-organismo e a determinação se essas proteínas são alvos para reconhecimento por anticorpos de pacientes. Nesse sentido, a abordagem de SEREX, baseada em bibliotecas de expressão construídas com proteínas recombinantes, vem sendo cada vez mais empregada como plataforma de descoberta de antígenos sororreativos.

Com o potencial de triar todos os antígenos de um patógeno, independentemente de sua abundância durante a infecção natural, os microarranjos de proteínas recombinantes são plataformas mais apropriadas para a descoberta de antígenos relacionados à resposta imune, proteção e progressão de determinada doença (Vigil et al., 2010). A principal vantagem dessa abordagem reside no fato de que virtualmente todas as proteínas do patógeno podem ser avaliadas individualmente, o que aumenta a chance de identificar proteínas que são expressas de forma transiente ou expressas em

menor quantidade, mas que podem ser utilizadas no desenvolvimento de novos mecanismos para intervenção imune, como ensaios diagnósticos, já que induzem uma resposta imune no paciente. No caso de organismos eucariotos, a biblioteca de expressão pode ser construída com base no perfil de RNAm do organismo em determinada condição (Kniemeyer et al., 2016).

Os microarranjos de proteínas recombinantes, porém, são limitados pela dificuldade em purificar a proteína recombinante, bem como pela complexidade dos sistemas de expressão gênica de alto desempenho, de modo que nem sempre é viável produzir e purificar as milhares de proteínas necessárias para representar o proteoma completo de um agente infeccioso. Além disso, a complexidade do enovelamento e da multimerização proteicas pode ser difícil de ser representada no microarranjo, assim como as modificações pós-traducionais que podem ser importantes para o reconhecimento antígeno-anticorpo (Vigil et al., 2010). Outra desvantagem da seleção e expressão de proteínas recombinantes por essa estratégia é o fato de que algumas proteínas podem não ser expressas ou ser expressas em quantidade insuficiente nos diferentes sistemas de expressão disponíveis atualmente, como procariotos (ex. *Escherichia coli*), leveduras e plantas.

Com o intuito de superar algumas dessas limitações, o grupo do Dr. Felgner desenvolveu uma metodologia de clonagem e expressão de proteínas recombinantes com alta eficiência e diminuição dos custos através de uma metodologia de alto desempenho, que emprega sistema de transcrição-tradução *in vitro* em sistema livre de células. Centenas a milhares de proteínas recombinantes são expressas no sistema livre de células e impressas em lâminas de microarranjo, que são utilizadas na captura de anticorpos específicos presentes no soro de indivíduos infectados ou outros animais. A quantidade de anticorpo capturada pode ser quantificada através do uso de um anticorpo secundário marcado (Davies et al., 2005). O grupo já aplicou essa metodologia a mais de 25 agentes de importância médica, incluindo vírus, bactérias, protozoários e helmintos e alguns dos antígenos identificados através dessa abordagem foram empregados com sucesso em diferentes

plataformas de diagnóstico (Barry et al., 2011; Liang et al., 2011; Trieu et al., 2011; Pinne et al., 2012; Tan et al., 2012). A metodologia se destaca por permitir um entendimento abrangente e em alta escala sobre a resposta imune humoral à infecção e foi empregada no presente trabalho para o estudo da leptospirose e da resposta imune desenvolvida por indivíduos naturalmente infectados.

2 OBJETIVOS

2.1 Objetivo Geral

- Investigar a resposta imune por anticorpos IgM e IgG em pacientes com diferentes formas clínicas de leptospirose, identificando antígenos marcadores de diagnóstico, prognóstico e proteção, através da plataforma de microarranjo de proteínas desenvolvida no Laboratório de Microarranjo de Proteínas da Universidade da Califórnia Irvine.

2.2 Objetivos Específicos

- Amplificação, clonagem e expressão das sequências que codificam para as proteínas preditas de *L. interrogans* sorovar Copenhageni;
- Confecção das lâminas de microarranjo para incubação com um painel de soros humanos;
- Realização da análise estatística dos dados gerados e seleção dos antígenos de interesse;
- Sub-clonagem e expressão, em sistema pET/*E. coli* BL21, dos genes referentes aos antígenos selecionados;
- Purificação, por cromatografia de afinidade, das proteínas expressas para avaliação do potencial diagnóstico em diferentes plataformas.

3 CAPÍTULO 1

Artigo intitulado *Proteomic Features Predict Seroreactivity against Leptospiral Antigens in Leptospirosis Patients*, publicado em 30 de outubro de 2014, na revista *Journal of Proteome Research* (DOI: 10.1021/pr500718t).

Neste estudo, foi produzido um microarranjo abrangendo 91% das proteínas preditas no genoma de *L. interrogans* sorovar Copenhageni para uma avaliação empírica de todos os antígenos reativos reconhecidos por soros de indivíduos infectados e/ou expostos ao patógeno. Cento e noventa e um antígenos induziram a formação de uma resposta imune por anticorpos IgM ou IgG, o que representa 5% do genoma codificante predito da bactéria. Estes antígenos foram categorizados de acordo com sua anotação gênica nos *Clusters of Orthologous Groups* (COG), realizada pelo NCBI e na *Mainrole Classification*, realizada pelo JCVI. Algumas características protéicas preditas computacionalmente, como presença de domínio transmembrana e localização subcelular, por exemplo, também foram avaliadas. Foram identificadas 14 categorias associadas ao reconhecimento por anticorpos, incluindo evidência de expressão por espectrometria de massas e de regulação positiva do RNAm. Em conjunto, estas categorias representam 25% do genoma codificante do patógeno e abrangem 50% dos antígenos reativos identificados. Portanto, este estudo forneceu não somente uma base empírica para a predição e classificação de antígenos sororreativos, como também uma maior compreensão acerca da resposta imune gerada durante a infecção natural por Leptospira.

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Proteomic Features Predict Seroreactivity against Leptospiral Antigens in Leptospirosis Patients

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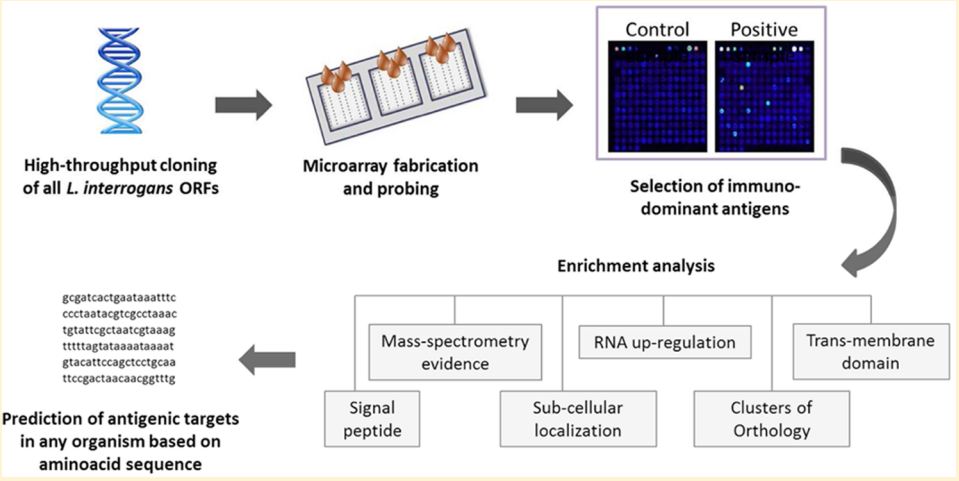
Supporting Information



ABSTRACT: With increasing efficiency, accuracy, and speed we can access complete genome sequences from thousands of infectious microorganisms; however, the ability to predict antigenic targets of the immune system based on amino acid sequence alone is still needed. Here we use a *Leptospira interrogans* microarray expressing 91% (3359) of all leptospiral predicted ORFs (3667) and make an empirical accounting of all antibody reactive antigens recognized in sera from naturally infected humans; 191 antigens elicited an IgM or IgG response, representing 5% of the whole proteome. We classified the reactive antigens into 26 annotated COGs (clusters of orthologous groups), 26 JCVI Mainrole annotations, and 11 computationally predicted proteomic features. Altogether, 14 significantly enriched categories were identified, which are associated with immune recognition including mass spectrometry evidence of in vitro expression and in vivo mRNA up-regulation. Together, this group of 14 enriched categories accounts for just 25% of the leptospiral proteome but contains 50% of the immunoreactive antigens. These findings are consistent with our previous studies of other Gram-negative bacteria. This genome-wide approach provides an empirical basis to predict and classify antibody reactive antigens based on structural, physical−chemical, and functional proteomic features and a framework for understanding the breadth and specificity of the immune response to *L. interrogans*.

KEYWORDS: *leptospirosis, protein microarray, enrichment analysis, antibody response*

INTRODUCTION

Leptospirosis is an emerging zoonotic disease of global importance and is now the leading cause of hemorrhagic disease. Humans are usually infected following contact with the urine of reservoir animals via contaminated soil or water.[1−3] While infection is asymptomatic in wild rodents and other reservoirs, in humans and other accidental hosts, it can cause severe clinical disease: hepato-renal failure (Weil's disease), pulmonary

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hemorrhage syndrome (LPHS), and even death. LPHS and Weil's disease have case fatalities of >50% and >10%, respectively.[2,4,5] The development of disease complications is thought to depend on infectious dose or host immunity. Protective immunity during naturally acquired infection is associated with a humoral immune response, indicated by increased production of agglutinating antibodies following infection and animal passive transfer studies.[6−10]

A major hurdle in the development of effective vaccines and serodiagnostic against complex microorganisms that encode thousands of proteins is the identification of a limited number of antigens that will induce a protective immune response. The advent of high-throughput sequencing during the past decade has made possible an approach called "reverse vaccinology",[11] which takes advantage of bioinformatics to narrow down the number of potential vaccine antigen candidates. Some proteomic features possibly associated with antigenicity and vaccine efficacy, such as extracellular location, outer-membrane proteins, signal peptides, and B- and T-cell epitopes, can be computationally predicted based on the amino acid sequence alone, reducing the number of candidates to several hundred. Although bioinformatic approaches can be used to remove groups of antigens from consideration as vaccine and serodiagnostic candidates, it tends to produce large lists of potential candidates that require additional expensive and time-consuming laboratory investigations to further narrow down the candidates. Bioinformatic approaches to antigen prediction are also inherently oversimplistic, and bona fide antigens can be missed.

More recently, whole proteome microarray chips, developed in our lab and by others, have provided an empirical approach to interrogate the entire proteome of any microorganism and to efficiently determine antibody profiles associated with acute or chronic infection or with vaccine mediated protection from infection.[12−14] Proteomic studies are a powerful tool for elucidating the molecular mechanisms involved in cellular functioning as well as disease development and progression. Protein microarray chips are particularly effective for the study of human serum samples, defining the antibody immune response against infectious agents on a proteomic scale and enabling the access to the complete antibody repertoire produced during an infection.[15,16] Additionally, the protein array usually encompasses thousands of proteins, representing the entire encoded proteome. The individual proteins printed on these chips capture antibodies in serum from infected individuals, which can be quantified using a fluorescent secondary antibody. In this fashion, antibodies produced after infection can be identified, characterizing the type of infection. The objective of proteomic studies is to provide a more complete understanding of the immune response to infection, also allowing the identification of novel serodiagnostic and prognostic markers as well as potential subunit vaccines.[14]

The mechanism for host recognition remains unclear, and the ability to predict antigenicity in silico is imperfect. Usually only a relatively small subset of the proteome of infectious agents, such as *L. interrogans*, is recognized by the immune system, and little is known about the characteristics of these antigens.[17] Consequently, our group has used proteome microarray data and enrichment analyses to identify proteomic features observed in the immunodominant and serodiagnostic antigen sets for more than 25 medically important agents and has successfully identified antigens that were later employed in a variety of diagnostic platforms.[13,15,18,19] Among the organisms studied there are viruses, protozoa, helminthes, and bacteria.[12,19−22] Here we have continued this iterative approach, first using a proteome microarray to identify the spectrum of immunoreactive *L. interrogans* antigens recognized in human leptospirosis cases and then classifying their reactivity according to annotated functional and computationally predicted features. These results inform us about the benefits and limitations of antigen prediction and provide a framework for future studies to improve predictive capability for serodominant antigens.

MATERIAL AND METHODS

Ethics Statement

The institutional review board committees of Yale University and Oswaldo Cruz Foundation approved the study protocol. Samples from infected patients came from the following projects: "Natural History of Urban Leptospirosis" (R01AI052473), "Disease Determinants of Urban Leptospirosis (U01AI088752), and "Ecoepidemiology of Leptospirosis" (R01TW009504). All participants provided written informed consent. After collection, a unique code identifier was assigned to each sample so that all samples were deidentified for researchers before their use.

Human Serum Samples

The study was conducted with a group of 90 laboratory-confirmed leptospirosis patients from the state of Bahia, Brazil, including 30 patients with mild clinical presentations, 31 patients with severe disease, and 30 patients who died due to leptospiral infection. Laboratory confirmation was defined based on the results of the microagglutination test (MAT) and according to the criteria of seroconversion or a four-fold rise in titer in patients with paired serum sample or a single titer of 1:800 in patients with only one serum sample. Sera samples from patients with severe leptospirosis were collected at three different time points and are designated as follows: (i) early acute sample, collected at patient admittance at the health care unit, (ii) late acute sample, collected 2 to 3 days after early acute sample collection, and (iii) convalescent sample, collected at least 14 days after the first sampling. For patients with mild leptospirosis, no late acute sample was collected; that is, only early acute and convalescent samples were provided. An early acute sample was collected from all deceased patients, but only five patients from this group survived through the late acute sampling. Samples were organized in groups, separated by clinical presentation (mild, severe, or deceased) and by time point (early acute, late acute, or convalescent) so that a total of 188 samples were categorized into seven groups.

Leptospira ORF Amplification and High-Throughput Cloning

The complete ORFeome of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was amplified by PCR and cloned into pXI vector using a high-throughput PCR recombination cloning method described elsewhere.[20,23] The cloning strategy allows the expression of recombinant proteins containing an N-terminal hemaglutinin (HA) tag and a C-terminal poly histidine (His) tag. Genes larger than 3 kb were cloned as smaller segments as previously described, and the *ligA* and *ligB* genes (LIC10465 and LIC10464, respectively) were fragmented according to the repeated Big domains present in the structure of each protein (LigB Repeats 7−12, LigA Repeats 7−13, and LigA/B Repeats 1−6),[24] which are recognized by human sera as previously described.[20] All PCR product sizes were confirmed by gel electrophoresis before cloning. Recombinant plasmids were confirmed by PCR using the insert specific primers for amplification. After identifying the seroreactive antigens on the microarrays, the inserts in the corresponding plasmids were confirmed by nucleotide sequencing by the Sanger method.

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Microarray Production and Probing

Microarray fabrication was performed as previously described.[20,23] In brief, purified mini-preparations of DNA were used for expression in a 10 μL *E. coli* in-vitro-based transcription-translation (IVTT) reaction system (RTS Kit, Roche) for 16 h at 26 °C with shaking (300 rpm) according to the manufacturer's instructions. Negative control reactions were those performed in the absence of DNA template ("NoDNA" controls). A protease inhibitor mixture (Complete, Roche) and Tween-20 (0.5% v/v final concentration) were added to the reactions to minimize protein degradation and improve protein solubilization. Unpurified supernatants were immediately printed onto nitrocellulose-coated glass FAST slides using an Omni Grid 100 microarray printer (Genomic Solutions) together with multiple negative control reactions and positive control spots of an IgG mix containing mouse, rat, and human IgG and IgM (Jackson Immuno Research).

Protein expression was verified by probing the array with monoclonal antipolyhistidine (Sigma-Aldrich) and antihemaglutinin (Roche Applied Science) diluted 1/400 in Protein Array Blocking Buffer (Whatman), as previously described.[20] Probing with human sera samples was performed with samples diluted 1/100 in Protein Array Blocking Buffer (Whatman) supplemented with *E. coli* lysate 10 mg/mL (McLab) at a final concentration of 10% v/v and incubated 30 min at room temperature with constant mixing. Antibodies bound to *E. coli* proteins were removed by centrifugation prior to addition to the microarray. Arrays were blocked for 30 min with Protein Array Blocking Buffer and then incubated with diluted samples overnight at 4 °C with gentle rocking. Washes and incubation with conjugate antibodies were performed as previously described.[20] Slides were scanned in a PerkinElmer ScanArray confocal laser and intensities were quantified using QuantArray package.

Genome Annotation and Computational Predictions

Enrichment analysis was performed based on *Leptospira interrogans* serovar Copenhageni L1-130 strain genome annotations available in the National Center for Biotechnology Information (NCBI) and John Craig Venter Institute (JCVI) databases. The antigens were classified according to annotated functional features and computationally predicted features. The clusters of orthologous groups (COG) information and the Mainrole Classification utilized can be found at NCBI and JCVI Web sites. The following programs were utilized for computational prediction: TMHMM v2.0 for trans-membrane domains prediction (http://www.cbs.dtu.dk/services/TMHMM/); SignalP v3.0 for signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/); and PSORTb v3.0 software for subcellular location prediction (http://www.psort.org/psortb/). Proteins with potentially biological importance, such as mass spectrometry (MS) evidence of in vitro expression[25] or in vivo mRNA up-regulation,[26] were also submitted to enrichment analysis.

Data Analysis

The reactivity was quantified using QuantArray software. Spot intensity raw data were obtained as the mean pixel signal intensity with automatic correction for spot-specific background. For each array, the average of control IVTT reactions (NoDNA controls) was subtracted from spot signal intensities to minimize background reactivity. Positive expression of proteins was determined by a signal intensity of 2.5 standard deviation (SD) above the mean of NoDNA control reactions for either the His or HA tags. The same cutoff was applied to identify the reactive proteins using the sera collection. Antigens were classified as seroreactive if (i) the average signal intensity of a sera group was above the established cutoff or (ii) at least 33% of the samples within a group showed individual signal intensity above the cutoff. Our rationale for using this second criterion was to include antigens that may not be strongly recognized by all of the patients but are recognized by a significant proportion of them. Enrichment statistical analysis was performed with Fisher's exact test.[12,19,22] When segments of fragmented proteins showed reactivity, enrichment analysis was performed considering the annotation/predictions of the original protein.

RESULTS

Gene Amplification, Cloning, and Protein Expression

We amplified the 3667 predicted ORFs from *L. interrogans* serovar Copenhageni from genomic DNA and cloned them into the pXi expression vector, as full or partial length proteins, using the high-throughput recombination cloning method developed by our group.[23] Cloning efficiency was ∼94%, and all cloned ORFs were expressed under a T7 promoter in the *E. coli* in vitro transcription/translation system. Microarray probing with anti-His and anti-HA antibodies revealed an expression level of ∼91% of all 3819 proteins and fragments printed on the array (3359 of 3667 total ORFs). A list of the proteins not represented on the microarrays is provided in Table S1 in the Supporting Information.

Overall Antibody Reactivity

We probed leptospiral protein arrays with a collection of 188 serum samples, composed of longitudinal samples from patients with mild and severe clinical presentations of leptospirosis, at different phases of the disease, as well as patients that died from acute leptospirosis infection. Analyzing longitudinal samples increased the likelihood of detecting an antigen with transient seroreactivity. In Figure 1, we show representative histograms of the number of reactive antigens selected from the convalescent time point for the severe patient group (n = 30) using the inclusion criteria (described in the Materials and Methods). For some antigens, the high average signal intensity is due to the strong reactivity of a few patients, as observed by the five orange dots below the orange dotted line in Figure 1B. We also observed that antigens with lower average signal intensity show positive reactivity in a few patients, as observed by the last 17 antigens in Figure 1A.

By applying these criteria to all groups of samples separately, we identified a total of 191 reactive antigens. Of these, 49 were reactive for both IgM and IgG antibodies, 61 were reactive only for IgM and, 81 were only for IgG (Figure 2). In this work, we investigated the proteomic features shared by antigens that are recognized by human sera because these features might increase their likelihood to be seroreactive. The aim is to identify all leptosprial antigens that are reactive in humans, without excluding cross-reactive antigens reactive in healthy unexposed individuals. A complete evaluation of the antibody immune response during naturally acquired leptospirosis compared with healthy controls, along with the analysis of new diagnostic and vaccine candidates, will be the focus of a separate study.

Functional Enrichment Analysis

L. interrogans serovar Copenhageni proteins are annotated with NCBI Clusters of Orthologous Groups (COG) functional categories (Table S2 in the Supporting Information). Each COG is defined by a group of three or more proteins that are inferred to be orthologs, that is, they are direct evolutionary counterparts

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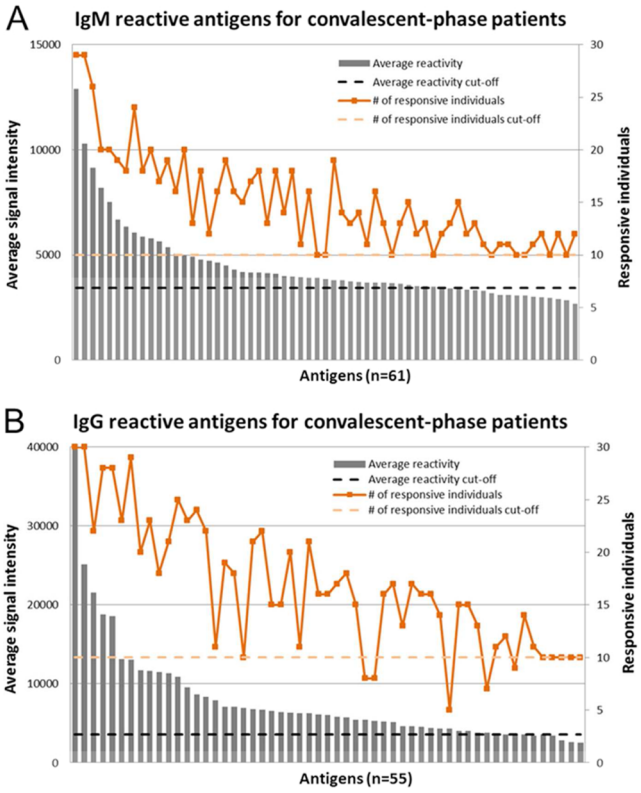

Figure 1. Representative histograms showing the criteria for selecting reactive antigens. An antigen was considered to be reactive if either the group average signal intensity or at least 33% of the samples within a group showed signal intensity above 2.5 standard deviations of the NoDNA control reactions. The histogram plots the average signal intensity (*Y* axis) and the number of responsive individuals (secondary *Y* axis) for each reactive antigen selected (*X* axis) for IgM (A) or IgG (B) probing of convalescent-phase samples from severe patients. Dotted lines correspond to the control reactions cutoff (black) or the minimum number of responsive individuals (orange) included using this criteria.

and represent a conserved functional category. Of the total 3667 leptospiral proteins, 1313 are not associated with COGs, whereas 500 proteins are associated with COGs but have not been assigned to a specific function, which are shown as general function prediction only (category R) or function unknown (category S). Additionally, some proteins have multiple category assignments. Using these definitions, we determined that there were 2416 COGs represented on the microarray.

Proteins with predicted COGs N (cell motility and secretion) and U (intracellular trafficking and secretion) were significantly enriched for both IgM and IgG reactivity, with significant fold increases of 4.4 (N) and 5.9 (U) for IgM and 3.8 (N) and 5.1 (U) for IgG, respectively (Table 1). We also found that proteins predicted as COG D (cell division and chromosome partitioning) were 4.5 times enriched in IgM responses. Interestingly, we determined that the IgG response to COGs S (unknown function) and V (defense mechanism) was 1.9- and 3.7-fold enriched, respectively, in IgG response, although these enrichments were less significant than COGs N and U. COG E (amino acid transport and metabolism) was lacking for IgG reactivity despite 4% of the *L. interrogans* ORFeome being classified in this category (Table 1).

Leptospiral proteins are also annotated with JCVI Mainrole Classification functional categories (Table S3 in the Supporting Information). Each Mainrole category can be divided into specific subrole categories. A total of 305 leptospiral proteins are not classified in any category, whereas 1257 proteins are assigned to a category but have no specific function prediction, shown as unknown function or hypothetical protein. Because some proteins have multiple functional assignments, we identified 3212 proteins predicted with JCVI Mainrole Classification in total for the 3667 proteins represented on the chip.

Similar to the COG categories enriched in patient sera, we found that proteins predicted as Cell Envelope and Cellular Processes were significantly enriched for both IgM and IgG reactivity, with fold increases of 2.2 and 3.7 for IgM and 2.3 and 3.0 for IgG, respectively (Table 1). We observed that antigens classified in the Protein Fate category in IgM responses, which is related to protein folding, stabilization, and degradation, were enriched 2.7-fold. Similarly to the COGs classifications, we found that proteins categorized as enzymes involved in Energy Metabolism were under-represented in the IgM response, with a fold decrease of 0.3 (Table 1). Interestingly, the JCVI class of proteins that comprised most of the reactive antigens was the Hypothetical Proteins category, with 23 and 29% of IgM- and IgG-reactive antigens, respectively.

We also analyzed the enrichment of antigens using computationally predicted features to identify motifs that are enriched in the humoral immune response to *Leptospira* infection (Tables S4−S6 in the Supporting Information). As shown in Table 1, we found that proteins containing 1 transmembrane domain were highly significantly enriched for both IgM and IgG antibodies reactivity, with fold increases of 2.0 and 1.7, respectively. Indeed, we observed that proteins lacking transmembrane domains were an under-represented feature in both responses, with fold decreases of 0.8 (IgM) and 0.4 (IgG). Interestingly, we observed that proteins with more than one predicted transmembrane domain were under-represented in IgG responses (0.3 fold change). Conversely, we determined that proteins with predicted signal peptides were significantly enriched in both IgM and IgG responses, with fold increases of 2.3 and 2.4, respectively. Accordingly, outer membrane proteins were also significantly enriched in both responses (3.2 and 4.1 fold increase), and proteins predicted as cytoplasmic localization were significantly under-represented (0.8 and 0.6 fold change). Finally, prediction of subcellular localization as extracellular was enriched for IgG reactivity, with a fold increase of 2.7. Although the majority of the antibody reactive antigens lack both a signal peptide and a transmembrane domain or are classified as cytoplasmic by pSort, these groups account for a larger proportion of all of the computationally classified proteins in the *L. interrogans* proteome. Consequently, they are under-represented by the enrichment analysis.

Antibody Reactivity versus Previous in Vitro and in Vivo Studies

Another potential characteristic that could influence protein antigenicity is the in vivo expression level because leptospiral proteins expressed at higher levels have a higher probability of being recognized by the host immune system. Therefore, we interfaced our seroreactive data with two existing data sets: (i) MS data obtained from in vitro cultured bacteria and (ii) a transcriptome study that identified differentially expressed genes when the bacteria was grown in vivo in dialysis membrane chambers (DMCs) implanted in rats. More than 50% (102/191) of the seroreactive antigens identified in our study were identified in vitro by MS (Figure 3A), making this method an enriching feature for both IgM and IgG seroreactivity (Table 1).

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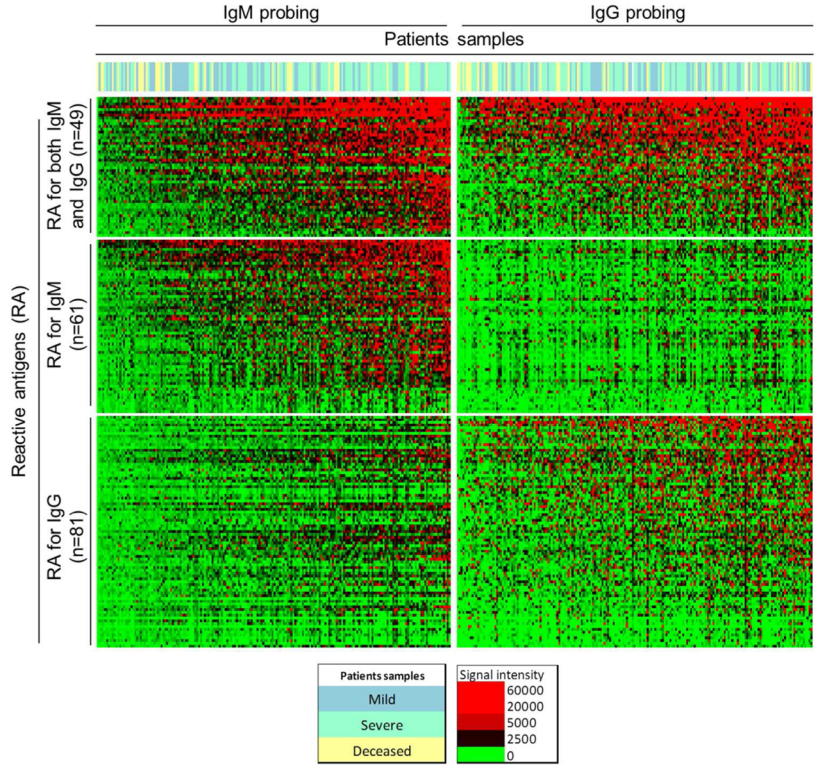

Figure 2. Heat map showing the overall IgM and IgG reactivity detected for mild, severe, and deceased patients. Reactivity intensity is shown according to the colorized scale. Antigens are shown in rows, grouped as reactive for both IgM and IgG antibodies, or for IgM- or IgG-only antibodies; patients are in columns, organized from left to right by the increasing average reactivity detected for the reactive antigens. Samples from patients with mild leptospirosis are shown in green, patients with severe presentations are in yellow, and deceased patients are in blue.

Table 1. Enriched and Under-Represented Proteomic Features Identified for IgM or IgG Reactivity

	IgM		IgG	
categories	FoldEnrich	*p* value	FoldEnrich	*p* value
Enriched				
COG U - intracellular trafficking and secretion[a]	5.9	1.49×10^{-7}	5.1	9.85×10^{-7}
presence of signal peptide[a]	2.3	5.00×10^{-7}	2.4	1.92×10^{-9}
JCVI - cellular processes[a]	3.7	4.11×10^{-6}	3.0	1.27×10^{-4}
COG N - cell motility and secretion[a]	4.4	1.12×10^{-5}	3.8	5.67×10^{-5}
1 trans-membrane domain[a]	2.0	8.67×10^{-5}	1.7	4.44×10^{-07}
JCVI - cell envelope[a]	2.2	4.96×10^{-4}	2.3	4.34×10^{-05}
mass spec positive[a]	1.4	1.19×10^{-3}	1.3	8.63×10^{-3}
outer membrane[a]	3.2	9.97×10^{-3}	4.1	2.61×10^{-4}
up-regulated mRNA in vivo[a]	2.5	1.48×10^{-2}	2.9	1.25×10^{-3}
JCVI - protein fate[b]	2.7	2.40×10^{-3}	1.9	5.76×10^{-2}
COG D - cell division and chromosome partitioning	4.5	2.67×10^{-2}	1.3	5.46×10^{-1}
extracellular[b]	1.6	4.37×10^{-1}	2.7	2.22×10^{-2}
COG S - function unknown[c]	0.8	8.32×10^{-1}	1.9	2.64×10^{-2}
COG V - defense mechanisms[c]	0.9	1.00×10^{0}	3.7	9.85×10^{-3}
Under-Represented				
lack of signal peptide[a]	0.8	5.00×10^{-7}	0.8	1.92×10^{-9}
0 trans-membrane domain[a]	0.8	8.21×10^{-5}	0.4	1.50×10^{-9}
cytoplasmic[a]	0.8	1.94×10^{-2}	0.6	1.31×10^{-5}
JCVI - energy metabolism[b]	0.3	4.56×10^{-2}	0.4	6.28×10^{-2}
COG E - amino acid transport and metabolism[c]	0.2	8.94×10^{-2}	0	6.17×10^{-3}
>1 trans-membrane domains[c]	1.2	4.08×10^{-1}	0.3	1.47×10^{-2}

[a]Significant features for both IgM and IgG antibodies. [b]Significant features for IgM. [c]Significant features for IgG.

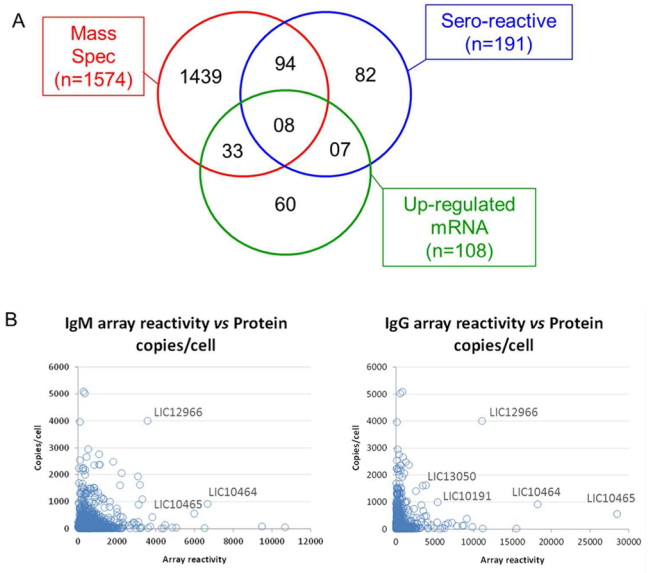

Figure 3. Correlation between the array seroreactivity and previously published studies. (A) Venn diagram of the leptospiral proteins identified by mass spectrometry when the bacteria were grown in vitro (red); proteins for which the corresponding mRNA was up-regulated when the bacteria were cultivated in dialysis membrane chambers in vivo (green); and antibody reactivity in leptospirosis patients detected by protein microarray (blue). (B) Scatter plots with the antibody reactivity and number of protein copies/cell detected by mass spectrometry. Array average signal intensity for IgM or IgG probing is plotted on the *X* axis; the number of protein copies per cell detected in vitro by mass spectrometry is shown on the *Y* axis.

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The up-regulated leptospiral genes identified in vivo were also enriched among the seroreactive antigens, with a fold change of 2.5 and 2.9 for IgM and IgG, respectively (Table 1).

Even though we observed a significant overlap between the number of seroreactive antigens identified in this study and the number of proteins detected in vitro by MS, there was no linear correlation between the number of protein copies per bacterium cell and the intensity of antibody reactivity detected on the protein arrays (Figure 3B). Few proteins had both high cellular concentrations and showed strong antibody response, as exemplified by LIC12966, LIC13050, LIC10191, and the Lig proteins (LIC10464 and LIC10465) in Figure 3B. Notably, >60% (67/108) of the up-regulated genes in vivo were not detected by MS under regular cultivation conditions, and only 15/108 up-regulated genes were also serodominant.

DISCUSSION

A major component of the adaptive immune response to infection is the generation of protective and long-lasting humoral immunity, but factors governing selection of the antigens recognized by the immune system are largely unknown.[17,19,27,28] It is not uncommon for viruses encoding a small number of proteins to elicit antibody responses against each structural protein. In contrast, for infectious bacteria or parasites that encode hundreds or thousands of proteins, only a subset of the proteome is recognized by the humoral immune response.[14,16,19,27,29,28] Here we describe utilization of a *Leptospira interrogans* proteome microarray to determine empirically the entire antibody repertoire of infected individuals. This technique allows the identification of the types of proteomic structural, physical−chemical, and functional features that are recognized more frequently by the human immune system. We previously identified a set of serodiagnostic antigens from a partial *L. interrogans* proteome.[20] An important difference between the present study and this previous report is that analysis of the complete proteome not only has allowed us to delineate additional serodiagnostic antigens but, more fundamentally, also has provided the basis for a rigorous, comprehensive, and quantitative determination of the protein characteristics of the entire set of the serodominant antigens on a genomic scale.

Our results represent a large-scale evaluation of *L. interrogans* proteins that are antigenic in the context of naturally acquired human infection. Our group has previously shown that >90% of the urban leptospirosis cases in Brazil are caused by *L. interrogans* serovar Copenhageni and has isolated strain L1-130 from an infected patient from the city of Salvador, Brazil.[30,31] The homogeneity of pathogen exposure and the use of a bacterial strain isolated from our study site minimize possible errors due to the natural diversity of *Leptospira* strains. Additionally, the availability of strain L1-130 complete genome sequence makes it ideal for this proteomic study.

We identified 191 immunodominant protein antigens with either IgM or IgG recognition, which correspond to ∼6% of the leptospiral coding genome. This relatively small set of antigens represents the complete repertoire of antibodies that are generated during symptomatic leptospiral infections. Mounting an immune response against a limited set of antigens may have the advantage of minimizing energy consumption and avoiding an excessive innate inflammatory reaction or cross-reactive autoimmune responses while still controlling infection effectively.

We classified the annotated functional proteomic features that are associated with antigenicity in humans. Enrichment analysis found 14 protein features that were enriched in the seroreactive antigens, of which 9 had both IgM and IgG responses (Table 1). Interestingly, we identified features that were significantly enriched exclusively in IgM or in IgG responses. Accounting for these differences in enrichment categories between IgG and IgM antigen targets may be related to class switching from IgM to IgG and affinity maturation of the IgG. The most significant enriched or under-represented features, however, were the ones detected for both IgM and IgG antibodies.

Similar studies from our group with *Brucella melitensis* and *Burkholderia pseudomallei* corroborate the enriching features identified for *Leptospira* (Table 1).[29,19] The most significant positive predictive features identified in this study, a signal peptide, 1 transmembrane domain, COG U (intracellular trafficking and secretion), and COG N (cell motility and secretion), were also enriched for *B. melitensis*. Accordingly, artemis-defined surface proteins (outer membrane, inner membrane, secreted, and surface structures) were enriched for *B. pseudomallei*. The enriched NCBI COG categories N and U are both related to protein secretion, and the externalization of such proteins can easily explain their accessibility to the host immune system. The presence of a signal peptide is a cellular tag for secretion or anchoring a protein on membranes, as we also found that proteins with one or more transmembrane domains, as well as outer-membrane subcellular location, were enriched. Cytoplasmic proteins were significantly under represented for all three pathogens. These studies demonstrate that antigens are not randomly recognized by the host immune response. Rather, the immune system focuses on specific types of antigens. Armed with this information and the increasing availability of genome sequences, we can begin to make generalizations to make a priori predictions about immunoreactivity of antigens based on amino acid sequence data alone.[32]

Sequence analysis and bioinformatics can be used to increase the odds of predicting immunoreactivity, but each has limitations that can be addressed by proteomic studies. For example, many reactive antigens cannot yet be predicted using bioinformatic methods because they do not contain features of an enrichment category, decreasing prediction sensitivity. In this study, we observed that 25% percent of the proteome is represented by enrichment categories, but only 12% of these proteins are seroreactive antigens. Furthermore, 50% of the reactive antigens identified here do not fall into any enrichment category and therefore cannot be predicted using this method. Use of the proteome microarray therefore allows the identification of a broader spectrum of reactive antigens that cannot be predicted by current methods; by selecting 25% of the proteome based on enriched categories, we would identify ∼50% of the immunoreactive antigens.

Proteomics is a powerful method for identifying seroreactive antigens but does have several limitations. For studies of this kind, we take advantage of bioinformatics tools to characterize the complete ORFeome of *L. interrogans* regarding proteomic features that help predict protein antigenicity in humans. Our findings are dependent on computational predictions and genome annotations. Even though different versions of the signal peptide predictor used here (SignalP v3 and v4) led to the same conclusions about enrichment (data not shown), we recognize that the use of different bioinformatics tools or possible errors and biases in the algorithms used can significantly alter the enrichment analysis. It is also possible that incorrect folding of the proteins printed on the microarrays may interfere with antigen−antibody recognition, either positively or negatively, and the accurate identification of immune-reactive proteins. Moreover, 9% of the

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leptospiral ORFeome were not represented on the microarrays, and we cannot affirm that those proteins are not seroreactive.

Our results correlate antigenicity with in vivo and in vitro expression of individual proteins allowing comparison of microarray data with data from previous protein expression studies. We found that >60% of the antigenic proteins were also detected by MS, indicating a large overlap in proteins expressed during both infection and in vitro growth. However, we identified no correlation between protein cellular concentration and seroreactivity intensity. Only 102 of the 1574 proteins identified by MS were found to be antigenic, suggesting that antigenicity involves structural features in addition to protein abundance. This discrepancy may be partially explained by differences between the level and identity of proteins in vitro and in vivo in the human host.

A similar argument can be made for the 93/108 differentially expressed leptospiral genes identified during cultivation in DMCs in rats that were not seroreactive on the array. For example, they identified eight up-regulated genes associated with virulence, of which four were identified as seroreactive in our study: LIC12631 and LIC12632 (hemolysins), LIC10465 (LigA), and LIC11219 (peroxiredoxin). Additionally, we identified 18 antigens associated with secretion (COG U) that could be involved in virulence. Of these, only LIC10053 was up-regulated in *Leptospira* in the rat reservoir. The profile of up-regulated leptospiral proteins may differ significantly between chronic carriage in an animal reservoir and the human host because of different host environments and immune responses. This lack of correlation between protein concentration and antigenicity supports the observation that up-regulated genes are not necessarily antigenic and that not all virulence factors elicit an antibody response.

Seroreactive antigens were selected based on a limited sera collection that comprised leptospirosis patients with different clinical outcomes from the city of Salvador in Bahia, Brazil. Expanding this collection or studying individuals from different areas, infected with different species or serovars, may provide an expanded set of reactive antigens. This study of specimens derived from one clinical site in Salvador involving exposure to one *Leptospira interrogans* strain has identified a reference set of antigens that are important in the humoral response to *Leptospira* infection in humans, including hypothetical proteins that would be missed by other bioinformatics approaches. Future studies comparing humoral responses among disease outcomes and against healthy, uninfected controls may identify prognostic markers and promising subunit vaccine candidates.

■ ASSOCIATED CONTENT

Supporting Information

Table S1. Proteins not represented on the proteome array. Table S2. COGs enrichment table. Table S3. JCVI main role enrichment table. Table S4. Trans-membrane domain enrichment table. Table S5. Signal peptide enrichment table. Table S6. Subcellular localization enrichment table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): P.L.F. receives income from Antigen Discovery, Inc., which is developing products related to the research described in this paper. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict of interest policies.

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Supplementary tables

Table S1. Proteins not represented on the proteome array

ORF ID	Accession	JCVI main role annotation	COG	Signal peptide prediction	Number of TM domains	Sub-cellular localization
LIC10889	YP_000866.1	Cell envelope	M	No	1	Unknown
LIC11008	YP_000981.1	Protein fate	M	No	2	Cytoplasmic
LIC11109	YP_001079.1	Unclassified	-	No	0	OuterMembrane
LIC11352	YP_001316.1	Unclassified	-	Yes	1	Unknown
LIC11364	YP_001328.1	Energy metabolism	R	No	0	Cytoplasmic
LIC11885	YP_001834.1	Cell envelope	-	Yes	0	Unknown
LIC12227	YP_002162.1	Purines, pyrimidines, nucleosides, and nucleotides	-	Yes	0	Unknown
LIC12322	YP_002256.1	Energy metabolism	I	No	0	Cytoplasmic
LIC13131	YP_003039.1	Hypothetical proteins	-	Yes	0	Unknown
LIC20250	YP_003634.1	Cell envelope	M	No	1	OuterMembrane
LIC20254	YP_003638.1	Regulatory functions	TK	No	0	Cytoplasmic
LIC10415	YP_000401.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC20044	YP_003436.1	Protein fate	O	No	0	Cytoplasmic
LIC13136	YP_003044.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC11791	YP_001743.1	DNA metabolism	KL	No	0	Cytoplasmic
LIC10772	YP_000752.1	Hypothetical proteins	-	Yes	1	Unknown
LIC10037	YP_000037.1	Central intermediary metabolism	E	No	0	Unknown
LIC11499	YP_001460.1	Hypothetical proteins	-	No	0	Unknown
LIC11898	YP_001847.1	Energy metabolism	C	No	0	Cytoplasmic

LIC10958	YP_000932.1	Energy metabolism	CR	No	0	Cytoplasmic
LIC11620	YP_001578.1	DNA metabolism	L	No	0	Cytoplasmic
LIC11707	YP_001659.1	Energy metabolism	G	No	0	Cytoplasmic
LIC10838	YP_000815.1	Energy metabolism	R	No	0	Cytoplasmic
LIC12414	YP_002345.1	Hypothetical proteins	-	No	0	Unknown
LIC12958	YP_002871.1	Hypothetical proteins	-	No	0	Unknown
LIC11423	YP_001385.1	Transcription	R	No	0	Unknown
LIC12744	YP_002664.1	Energy metabolism	C	No	0	Cytoplasmic
LIC12251	YP_002186.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC13194	YP_003102.1	Energy metabolism	E	No	0	Unknown
LIC11500	YP_001461.1	Protein fate	J	No	0	Cytoplasmic
LIC10712	YP_000696.1	Energy metabolism	C	No	1	Unknown
LIC11851	YP_001800.1	Protein synthesis	-	Yes	1	OuterMembrane
LIC12679	YP_002601.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC11854	YP_001803.1	Cell envelope	R	No	0	Cytoplasmic
LIC11989	YP_001928.1	Unclassified	G	Yes	1	Unknown
LIC10163	YP_000155.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10241	YP_000232.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC11514	YP_001473.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12331	YP_002263.1	Energy metabolism	G	Yes	0	Cytoplasmic
LIC12675	YP_002597.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC13261	YP_003168.1	Cell envelope	R	No	0	Cytoplasmic
LIC20156	YP_003547.1	Unclassified	S	No	0	Unknown
LIC11134	YP_001104.2	Central intermediary metabolism	BQ	No	0	Cytoplasmic
LIC10434	YP_000419.1	Hypothetical proteins	-	Yes	0	Unknown
LIC10723	YP_000707.1	Cellular processes	N	No	0	Periplasmic

LIC11062	YP_001032.1	Central intermediary metabolism	-	No	0	Cytoplasmic
LIC20192	YP_003583.1	Unclassified	R	Yes	0	Extracellular
LIC11433	YP_001395.1	Regulatory functions	T	No	0	Cytoplasmic
LIC13032	YP_002945.1	Hypothetical proteins	-	No	1	Unknown
LIC12646	YP_002569.1	Protein fate	M	No	1	CytoplasmicMembrane
LIC10298	YP_000288.1	Cellular processes	N	No	0	Periplasmic
LIC12993	YP_002906.1	Hypothetical proteins	-	Yes	1	CytoplasmicMembrane
LIC10021	YP_000021.1	Cell envelope	-	Yes	0	Unknown
LIC10137	YP_000133.1	Unclassified	O	No	0	Unknown
LIC10417	YP_000403.1	Hypothetical proteins	-	No	0	Unknown
LIC10774	YP_000754.1	Cell envelope	-	Yes	0	Unknown
LIC10784	YP_000763.1	No Data	-	No	0	Unknown
LIC10785	YP_000764.1	Mobile and extrachromosomal element functions	L	No	0	Unknown
LIC10865	YP_000842.1	Unclassified	S	Yes	1	Unknown
LIC11033	YP_001003.1	Hypothetical proteins	-	No	1	Unknown
LIC11225	YP_001194.1	No Data	-	No	0	Unknown
LIC11242	YP_001211.1	Energy metabolism	C	No	0	Cytoplasmic
LIC11305	YP_001271.1	Hypothetical proteins	H	No	0	Cytoplasmic
LIC11306	YP_001272.1	Hypothetical proteins	S	No	1	Unknown
LIC11321	YP_001287.1	Hypothetical proteins	-	Yes	1	Unknown
LIC11382	YP_001346.1	Hypothetical proteins	-	No	0	Unknown
LIC11432	YP_001394.1	Regulatory functions	T	No	0	CytoplasmicMembrane
LIC11587	YP_001546.1	Purines, pyrimidines, nucleosides, and nucleotides	F	No	0	Cytoplasmic
LIC11690	YP_001642.1	Hypothetical proteins	-	Yes	1	Unknown
LIC11760	YP_001712.1	Protein synthesis	J	No	0	Cytoplasmic
LIC11995	YP_001934.1	Hypothetical proteins	-	No	0	Unknown
LIC12111	YP_002045.2	Protein synthesis	J	No	0	Cytoplasmic

LIC12159	YP_002094.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12249	YP_002184.1	Hypothetical proteins	-	No	1	Cytoplasmic
LIC12385	YP_002317.1	No Data	-	No	1	Unknown
LIC12525	YP_002453.1	Cell envelope	-	Yes	0	Unknown
LIC12558	YP_002485.1	Cell envelope	-	Yes	0	Unknown
LIC12637	YP_002560.1	Protein synthesis	J	No	0	Cytoplasmic
LIC12839	YP_002755.1	Purines, pyrimidines, nucleosides, and nucleotides	F	No	0	Cytoplasmic
LIC12855	YP_002771.1	Protein synthesis	J	No	0	Unknown
LIC12860	YP_002776.1	No Data	J	No	0	Cytoplasmic
LIC12865	YP_002781.1	Protein synthesis	-	No	0	Unknown
LIC13013	YP_002926.1	No Data	-	No	0	Unknown
LIC13034	YP_002947.1	No Data	-	No	0	Unknown
LIC13109	YP_003018.1	Hypothetical proteins	R	No	0	Cytoplasmic
LIC13163	YP_003071.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC13182	YP_003090.1	Energy metabolism	C	No	0	Cytoplasmic
LIC13221	YP_003129.1	Energy metabolism	O	No	0	Cytoplasmic
LIC13391	YP_003294.1	No Data	-	No	0	Unknown
LIC13422	YP_003325.1	Energy metabolism	CP	No	12	CytoplasmicMembrane
LIC14000	YP_003858659.1		-	No	0	Unknown
LIC20065	YP_003457.1	Fatty acid and phospholipid metabolism	IQ	No	0	Cytoplasmic
LIC20263	YP_003647.1	No Data	-	Yes	0	CytoplasmicMembrane
LIC20300	YP_003858670.1		-	No	0	Unknown
LIC12693	YP_002614.1	Cell envelope	MU	Yes	0	Unknown
LIC13301	YP_003206.1	Fatty acid and phospholipid metabolism	I	No	0	Unknown
LIC10583	YP_000567.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC13009	YP_002922.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic

LIC10445	YP_000430.1	Central intermediary metabolism	M	No	0	Cytoplasmic
LIC11571	YP_001530.1	Protein fate	NU	No	0	Cytoplasmic
LIC20175	YP_003566.1	Protein fate	T	No	0	Cytoplasmic
LIC12026	YP_001964.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC12535	YP_002463.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC11602	YP_001560.1	Unclassified	G	No	0	Unknown
LIC13020	YP_002933.2	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10641	YP_000625.1	Fatty acid and phospholipid metabolism	T	No	0	CytoplasmicMembrane
LIC20259	YP_003643.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10485	YP_000469.1	Unclassified	-	No	0	Cytoplasmic
LIC13110	YP_003019.1	Regulatory functions	T	No	0	CytoplasmicMembrane
LIC12700	YP_002621.1	Protein fate	R	No	0	Unknown
LIC12163	YP_002098.1	Purines, pyrimidines, nucleosides, and nucleotides	O	No	0	Cytoplasmic
LIC12785	YP_002702.1	Unknown function	R	No	0	Cytoplasmic
LIC12302	YP_002236.1	Protein fate	O	No	0	Extracellular
LIC13280	YP_003187.1	Unknown function	I	No	0	Unknown
LIC10668	YP_000652.1	Unclassified	V	No	0	Cytoplasmic
LIC13457	YP_003360.1	Hypothetical proteins	-	No	3	CytoplasmicMembrane
LIC10561	YP_000545.1	Hypothetical proteins	-	Yes	0	Extracellular
LIC11773	YP_001725.1	Amino acid biosynthesis	E	No	0	Cytoplasmic
LIC10376	YP_000362.1	Hypothetical proteins	R	No	3	CytoplasmicMembrane
LIC10454	YP_000438.1	Hypothetical proteins	R	No	0	Cytoplasmic
LIC13389	YP_003292.1	DNA metabolism	L	No	3	CytoplasmicMembrane
LIC12428	YP_002359.1	Transport and binding proteins	V	No	0	CytoplasmicMembrane
LIC12692	YP_002613.1	Purines, pyrimidines, nucleosides, and	O	No	0	Cytoplasmic

		nucleotides				
LIC10139	YP_000135.1	Fatty acid and phospholipid metabolism	T	No	0	Cytoplasmic
LIC10154	YP_000147.1	Protein synthesis	J	No	0	Cytoplasmic
LIC10309	YP_000299.1	Energy metabolism	E	No	0	Cytoplasmic
LIC10689	YP_000673.1	Energy metabolism	P	No	0	Cytoplasmic
LIC10767	YP_000747.1	Hypothetical proteins	-	No	0	Unknown
LIC10821	YP_000798.1	Cell envelope	-	Yes	0	Unknown
LIC10882	YP_000859.1	Hypothetical proteins	-	No	0	OuterMembrane
LIC10943	YP_000917.1	Mobile and extrachromosomal element functions	L	No	0	Cytoplasmic
LIC10998	YP_000972.1	Hypothetical proteins	-	No	0	OuterMembrane
LIC11172	YP_001142.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC11215	YP_001184.1	No Data	-	No	0	Unknown
LIC11243	YP_001212.1	Energy metabolism	C	No	0	Cytoplasmic
LIC11502	YP_001463.1	Hypothetical proteins	-	No	0	Unknown
LIC11540	YP_001499.1	Purines, pyrimidines, nucleosides, and nucleotides	F	No	0	Cytoplasmic
LIC11827	YP_001778.1	Protein fate	M	No	0	Cytoplasmic
LIC11867	YP_001816.1	Cell envelope	M	No	0	Cytoplasmic
LIC12234	YP_002169.1	Transport and binding proteins	-	No	0	Extracellular
LIC12462	YP_002390.1	Protein synthesis	J	No	0	Cytoplasmic
LIC12472	YP_002400.1	Central intermediary metabolism	-	No	0	Cytoplasmic
LIC12634	YP_002557.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12711	YP_002632.1	No Data	E	No	0	Cytoplasmic
LIC12912	YP_002828.1	Mobile and extrachromosomal element functions	-	No	0	Cytoplasmic
LIC12914	YP_002830.1	No Data	L	No	0	Unknown
LIC12970	YP_002883.1	Cellular processes	D	No	0	Cytoplasmic
LIC13148	YP_003056.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC13177	YP_003085.1	DNA metabolism	-	No	0	Cytoplasmic

LIC13252	YP_003159.1	Energy metabolism	C	No	0	Cytoplasmic
LIC13305	YP_003210.1	Cell envelope	-	Yes	0	Unknown
LIC13349	YP_003254.1	Hypothetical proteins	-	No	1	Unknown
LIC13427	YP_003330.1	No Data	T	No	1	Unknown
LIC20180	YP_003571.1	Regulatory functions	T	No	0	CytoplasmicMembrane
LIC12357	YP_002289.1	Unknown function	T	No	0	CytoplasmicMembrane
LIC12616	YP_002539.1	Hypothetical proteins	-	No	0	Unknown
LIC10396	YP_000382.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC10522	YP_000506.1	Energy metabolism	R	No	0	Cytoplasmic
LIC20209	YP_003598.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC11555	YP_001514.2	Protein synthesis	J	No	0	Cytoplasmic
LIC12551	YP_002478.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC12027	YP_001965.1	Fatty acid and phospholipid metabolism	Q	No	0	Cytoplasmic
LIC10538	YP_000522.1	Transport and binding proteins	R	No	0	Cytoplasmic
LIC13219	YP_003127.2	Protein synthesis	J	No	0	Cytoplasmic
LIC12792	YP_002709.1	Central intermediary metabolism	K	No	0	Cytoplasmic
LIC11067	YP_001037.1	Unknown function	M	No	0	Unknown
LIC10344	YP_000334.1	Transcription	T	No	0	Unknown
LIC11741	YP_001693.1	Regulatory functions	R	No	0	Cytoplasmic
LIC10222	YP_000213.1	DNA metabolism	L	No	0	Cytoplasmic
LIC11536	YP_001495.1	Unclassified	M	Yes	0	Unknown
LIC13372	YP_003277.1	Central intermediary metabolism	EH	No	0	Cytoplasmic
LIC12173	YP_002108.1	Cell envelope	M	No	0	Cytoplasmic
LIC13464	YP_003367.1	Protein fate	E	No	0	Unknown
LIC13021	YP_002934.1	Hypothetical proteins	T	No	0	Unknown
LIC10662	YP_000646.1	Hypothetical proteins	-	Yes	1	CytoplasmicMembrane
LIC11811	YP_001762.1	Unclassified	M	No	0	Cytoplasmic
LIC13331	YP_003236.1	Regulatory functions	T	No	0	CytoplasmicMembrane

LIC12268	YP_002203.1	Energy metabolism	C	No	1	Cytoplasmic
LIC13147	YP_003055.1	Protein fate	R	No	0	Cytoplasmic
LIC12371	YP_002303.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC11844	YP_001793.1	Unclassified	O	No	2	Cytoplasmic
LIC10040	YP_000040.1	Cell envelope	M	No	0	Cytoplasmic
LIC10068	YP_000068.1	Hypothetical proteins	-	No	0	Unknown
LIC12600	YP_002523.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10678	YP_000662.1	Regulatory functions	TK	No	0	Cytoplasmic
LIC13077	YP_002989.1	Hypothetical proteins	R	No	0	Cytoplasmic
LIC11311	YP_001277.1	Unclassified	I	No	0	Unknown
LIC11421	YP_001383.1	Energy metabolism	P	No	11	CytoplasmicMembrane
LIC12014	YP_001953.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12298	YP_002232.1	Hypothetical proteins	-	No	0	Unknown
LIC11879	YP_001828.1	Hypothetical proteins	S	No	0	Cytoplasmic
LIC12178	YP_002113.1	Cell envelope	-	Yes	9	CytoplasmicMembrane
LIC13302	YP_003207.1	Hypothetical proteins	-	No	4	CytoplasmicMembrane
LIC12097	YP_002031.1	Regulatory functions	T	No	0	CytoplasmicMembrane
LIC12539	YP_002467.1	Hypothetical proteins	-	Yes	1	Extracellular
LIC10323	YP_000313.1	Energy metabolism	G	No	0	Cytoplasmic
LIC11045	YP_001015.1	Regulatory functions	T	No	0	Cytoplasmic
LIC11390	YP_001353.1	Cellular processes	NUO	No	0	Cytoplasmic
LIC11624	YP_001582.1	DNA metabolism	L	No	0	Cytoplasmic
LIC10051	YP_000051.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC20222	YP_003611.1	Hypothetical proteins	R	No	0	Cytoplasmic
LIC10950	YP_000924.1	Regulatory functions	TK	No	0	Cytoplasmic
LIC13218	YP_003126.1	Unclassified	R	No	0	Cytoplasmic
LIC10161	YP_000153.1	Hypothetical proteins	-	No	0	Cytoplasmic

LIC10227	YP_000218.1	Hypothetical proteins	S	No	0	Cytoplasmic
LIC13118	YP_003027.1	Cell envelope	M	No	0	Cytoplasmic
LIC10529	YP_000513.1	Unclassified	S	No	0	Cytoplasmic
LIC11748	YP_001700.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12019	YP_001957.1	Transcription	KL	No	0	Cytoplasmic
LIC10200	YP_000191.1	Protein fate	M	No	0	Cytoplasmic
LIC10131	YP_000127.1	Protein synthesis	D	No	0	Cytoplasmic
LIC10189	YP_000180.1	Hypothetical proteins	-	No	0	Unknown
LIC10083	YP_000082.1	Hypothetical proteins	-	No	3	CytoplasmicMembrane
LIC10217	YP_000208.1	No Data	-	No	2	CytoplasmicMembrane
LIC10255	YP_000246.1	Protein fate	O	No	0	Cytoplasmic
LIC10448	YP_000432.1	Protein synthesis	J	No	0	Cytoplasmic
LIC10563	YP_000547.1	Unclassified	R	No	1	CytoplasmicMembrane
LIC10639	YP_000623.1	Hypothetical proteins	-	Yes	0	Extracellular
LIC10688	YP_000672.1	Energy metabolism	-	No	3	CytoplasmicMembrane
LIC10762	YP_000742.1	Protein synthesis	J	No	0	Cytoplasmic
LIC10807	YP_000784.1	Central intermediary metabolism	O	No	0	Cytoplasmic
LIC10867	YP_000844.1	Hypothetical proteins	-	No	0	Unknown
LIC10917	YP_000892.1	Hypothetical proteins	-	No	0	Unknown
LIC10955	YP_000929.1	Biosynthesis of cofactors, prosthetic groups, and carriers	I	No	0	Cytoplasmic
LIC11002	YP_000975.1	Mobile and extrachromosomal element functions	-	No	0	Unknown
LIC11126	YP_001096.1	Unknown function	T	No	0	Cytoplasmic
LIC11178	YP_001148.1	Hypothetical proteins	-	Yes	0	Cytoplasmic
LIC11226	YP_001195.1	Hypothetical proteins	-	No	4	CytoplasmicMembrane
LIC11269	YP_001238.1	No Data	-	No	0	Unknown
LIC11308	YP_001274.1	Amino acid biosynthesis	E	No	0	Cytoplasmic
LIC11322	YP_001288.1	Hypothetical proteins	-	No	0	Unknown

LIC11323	YP_001289.1	DNA metabolism	-	No	0	Unknown
LIC11339	YP_001303.1	DNA metabolism	L	No	0	Cytoplasmic
LIC11497	YP_001458.1	Central intermediary metabolism		No	0	Cytoplasmic
LIC11717	YP_001669.1	DNA metabolism	L	No	0	Cytoplasmic
LIC11746	YP_001698.1	Amino acid biosynthesis	E	No	0	Cytoplasmic
LIC11774	YP_001726.1	DNA metabolism	L	No	0	Cytoplasmic
LIC12335	YP_002267.1	Purines, pyrimidines, nucleosides, and nucleotides	E	No	0	Cytoplasmic
LIC12370	YP_002302.1	Transcription	-	No	0	Unknown
LIC12386	YP_002318.1	Unclassified	GER	No	10	CytoplasmicMembrane
LIC12406	YP_002338.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12417	YP_002348.1	Unknown function	R	No	0	Cytoplasmic
LIC12461	YP_002389.1	Protein synthesis	J	No	0	Cytoplasmic
LIC12650	YP_002573.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC12670	YP_002592.1	Unclassified	T	No	2	CytoplasmicMembrane
LIC12688	YP_002609.1	DNA metabolism	L	No	0	Cytoplasmic
LIC12697	YP_002618.1	Hypothetical proteins	-	No	0	Unknown
LIC12750	YP_002670.1	Energy metabolism	CP	No	15	CytoplasmicMembrane
LIC12752	YP_002672.1	Energy metabolism	C	No	11	CytoplasmicMembrane
LIC12753	YP_002673.1	Hypothetical proteins	-	No	0	Unknown
LIC12784	YP_002701.1	Cell envelope	-	No	0	Unknown
LIC12825	YP_002741.1	Unclassified	E	No	0	Unknown
LIC12869	YP_002785.1	Protein synthesis	J	No	0	Cytoplasmic
LIC13081	YP_002993.1	Hypothetical proteins	-	No	0	Unknown
LIC13129	YP_003037.1	Protein synthesis	J	No	0	Cytoplasmic
LIC13291	YP_003198.1	Central intermediary metabolism	R	No	1	Unknown
LIC13304	YP_003209.1	Hypothetical proteins	-	No	0	Unknown
LIC13317	YP_003222.1	Hypothetical proteins	-	No	0	Cytoplasmic

LIC13320	YP_003225.1	Protein fate	E	No	0	Extracellular
LIC13322	YP_003227.1	Protein fate	E	Yes	0	Extracellular
LIC13430	YP_003333.1	Unclassified	G	No	0	Cytoplasmic
LIC13468	YP_003370.1	Hypothetical proteins	-	No	0	Unknown
LIC14005	YP_003858660.1		-	No	0	Unknown
LIC20255	YP_003639.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10382	YP_000368.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC10543	YP_000527.1	Fatty acid and phospholipid metabolism	I	No	0	Cytoplasmic
LIC11173	YP_001143.1	Unknown function	L	No	0	Cytoplasmic
LIC12505	YP_002433.1	Regulatory functions	T	No	0	CytoplasmicMembrane
LIC12762	YP_002682.1	Hypothetical proteins	T	No	0	Cytoplasmic
LIC20198	YP_003587.1	Hypothetical proteins	T	No	0	Cytoplasmic
LIC11257	YP_001226.1	Central intermediary metabolism	FP	No	0	Cytoplasmic
LIC12350	YP_002282.1	Protein synthesis	J	No	0	Cytoplasmic
LIC13448	YP_003351.1	Regulatory functions	T	No	0	Unknown
LIC10174	YP_000166.1	Hypothetical proteins	-	No	0	Unknown
LIC10896	YP_000873.1	Transport and binding proteins	P	Yes	0	OuterMembrane
LIC20009	YP_003401.1	Biosynthesis of cofactors, prosthetic groups, and carriers	H	No	0	Cytoplasmic
LIC12701	YP_002622.1	Transcription	J	No	0	Cytoplasmic
LIC12421	YP_002352.1	Hypothetical proteins	R	No	0	Cytoplasmic
LIC12205	YP_002140.1	Unclassified	MG	No	0	Cytoplasmic
LIC11483	YP_001444.1	Unknown function	L	No	0	Cytoplasmic
LIC20088	YP_003480.1	Unclassified	G	No	0	Cytoplasmic
LIC10151	YP_000145.1	Hypothetical proteins	-	No	2	Cytoplasmic
LIC12536	YP_002464.1	Unclassified	H	No	0	Cytoplasmic
LIC10350	YP_000339.1	Hypothetical proteins	T	No	0	Unknown
LIC12413	YP_002344.1	Unclassified	S	No	0	Unknown

LIC20265	YP_003649.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC13308	YP_003213.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10322	YP_000312.1	Hypothetical proteins	-	No	2	Cytoplasmic
LIC10457	YP_000441.1	Hypothetical proteins	-	No	0	Cytoplasmic
LIC10556	YP_000540.1	Hypothetical proteins	-	No	0	Unknown
LIC11034	YP_001004.1	Hypothetical proteins	-	No	0	Unknown
LIC11246	YP_001215.1	Central intermediary metabolism	R	No	0	Extracellular
LIC11248	YP_001217.1	Energy metabolism	M	No	11	CytoplasmicMembrane
LIC11453	YP_001414.1	Amino acid biosynthesis	E	No	0	Cytoplasmic
LIC11460	YP_001421.1	Protein synthesis	J	No	0	Cytoplasmic
LIC11558	YP_001517.1	Protein synthesis	J	No	0	Cytoplasmic
LIC11647	YP_001605.1	Protein synthesis	J	No	0	Cytoplasmic
LIC11655	YP_001611.1	Purines, pyrimidines, nucleosides, and nucleotides	F	No	0	Cytoplasmic
LIC11869	YP_001818.1	Unknown function	M	No	0	Cytoplasmic
LIC12025	YP_001963.1	Protein synthesis	J	No	0	Cytoplasmic
LIC12562	YP_002489.1	Energy metabolism	PR	No	0	Cytoplasmic
LIC13404	YP_003307.1	Unclassified	I	No	0	Cytoplasmic
LIC20055	YP_003447.1	Hypothetical proteins	S	No	0	Cytoplasmic
LIC20110	YP_003502.1	Hypothetical proteins	-	Yes	1	Unknown
LIC10464	YP_000448.1	Unknown function	N	Yes	1	Unknown
LIC10465	YP_000449.1	Unknown function	-	Yes	0	Unknown
LIC11739	YP_001691.1	Hypothetical proteins	R	No	0	Unknown
LIC11026	YP_000996.1	Hypothetical proteins	-	No	1	OuterMembrane
LIC12602	YP_002525.1	Hypothetical proteins	-	No	0	Unknown
LIC11755	YP_001707.1	Hypothetical proteins	-	No	0	OuterMembrane
LIC12048	YP_001983.1	Hypothetical proteins	-	No	1	OuterMembrane

Table S2. COG enrichment

COG	Definition	Proteins on chip	IgM				IgG			
			Hits		FoldEnrich	p-value	Hits		FoldEnrich	p-value
A	RNA processing and modification	0	0		0.0	1.00E+00	0		0.0	1.00E+00
B	Chromatin structure and dynamics	2	0		0.0	1.00E+00	0		0.0	1.00E+00
C	Energy production and conversion	119	3		0.8	1.00E+00	1		0.2	1.30E-01
D	Cell division and chromosome partitioning	22	3	*	4.4	2.95E-02	1		1.3	5.55E-01
E	Amino acid transport and metabolism	152	1		0.2	9.08E-02	0	**	0.0	6.29E-03
F	Nucleotide transport and metabolism	49	1		0.7	1.00E+00	0		0.0	4.22E-01
G	Carbohydrate transport and metabolism	93	1		0.3	3.69E-01	1		0.3	2.61E-01
H	coenzyme metabolism	122	2		0.5	5.90E-01	3		0.7	8.02E-01
I	Lipid metabolism	103	1		0.3	3.78E-01	0		0.0	5.27E-02
J	Translation, ribosomal structure and biogenesis	148	2		0.4	3.29E-01	1		0.2	6.59E-02
K	Transcription	110	2		0.6	5.84E-01	2		0.5	4.38E-01
L	DNA replication, recombination and repair	135	1		0.2	1.29E-01	1		0.2	9.32E-02
M	Cell envelope biogenesis, outer membrane	217	5		0.7	6.85E-01	8		1.0	8.52E-01
N	Cell motility and secretion	90	12	*	4.3	1.69E-05	12	*	3.7	7.32E-05
O	Posttranslational modification, protein turnover, chaperones	103	5		1.6	2.53E-01	4		1.1	7.87E-01
P	Inorganic ion transport and metabolism	91	0		0.0	1.17E-01	2		0.6	7.73E-01
Q	Secondary metabolites biosynthesis, transport and catabolism	28	0		0.0	1.00E+00	0		0.0	6.24E-01
R	General function prediction only	302	5		0.5	1.65E-01	5		0.5	7.42E-02
S	Function unknown	198	5		0.8	8.33E-01	13	*	1.8	2.99E-02
T	Signal transduction mechanisms	220	5		0.7	6.87E-01	5		0.6	3.52E-01
U	Intracellular trafficking and secretion	73	13	*	5.7	2.41E-07	13	*	4.9	1.33E-06

V	Defense mechanisms	38	1	0.8	1.00E+00	5	*	3.7	1.09E-02
W	Extracellular Structure	0	0	0.0	1.00E+00	0		0.0	1.00E+00
Y	Nuclear structure	0	0	0.0	1.00E+00	0		0.0	1.00E+00
Z	Cytoskeleton	1	0	0.0	1.00E+00	0		0.0	1.00E+00
	Not in COGs	1469	53	1.2	1.82E-01	62		1.2	7.64E-02
	Total ORFs	3885	121			140			
	True positive rate	-		0.231				0.309	
	True negative rate	-		0.958				0.905	
	Positive predictive value	-		0.151				0.108	
	Negative predictive value	-		0.975				0.973	

*Enriched category. **Under represented category.

NOTE: Proteins may be included in more than one category.

Table S3. JCVI mainrole enrichme

JCVI mainrole	Proteins on chip	IgM				IgG			
		Hits		FoldEnrich	p-value	Hits		FoldEnrich	p-value
Amino acid biosynthesis	78	0		0.0	1.74E-01	0		0.0	1.14E-01
Biosynthesis of cofactors, prosthetic groups, and carriers	92	0		0.0	1.15E-01	1		0.3	3.79E-01
Cell envelope	322	21	*	2.2	4.61E-04	26	*	2.3	3.99E-05
Cellular processes	145	16	*	3.7	4.29E-06	15	*	2.9	1.32E-04
Central intermediary metabolism	74	2		0.9	1.00E+00	2		0.8	1.00E+00
Disrupted reading frame	0	0		0.0	1.00E+00	0		0.0	1.00E+00
DNA metabolism	91	1		0.4	5.25E-01	1		0.3	3.77E-01
Energy metabolism	228	2	**	0.3	6.63E-02	3		0.4	6.27E-02
Fatty acid and phospholipid metabolism	100	2		0.7	7.69E-01	1		0.3	2.64E-01
Gene/protein expression	0	0		0.0	1.00E+00	0		0.0	1.00E+00
Hypothetical proteins	1118	25		0.7	9.10E-02	38		1.0	8.46E-01
Hypothetical proteins - Conserved	0	0		0.0	1.00E+00	0		0.0	1.00E+00
Metabolism	0	0		0.0	1.00E+00	0		0.0	1.00E+00
Mobile and extrachromosomal element functions	60	0		0.0	2.63E-01	0		0.0	2.73E-01
Pathogen responses	0	0		0.0	1.00E+00	0		0.0	1.00E+00
Protein fate	137	11	*	2.7	2.32E-03	9		1.9	5.66E-02
Protein synthesis	169	7		1.4	3.51E-01	5		0.8	8.33E-01
Purines, pyrimidines, nucleosides, and nucleotides	62	1		0.5	1.00E+00	0		0.0	1.72E-01
Regulatory functions	192	3		0.5	3.78E-01	2		0.3	6.60E-02
Signal transduction	0	0		0.0	1.00E+00	0		0.0	1.00E+00
Transcription	61	1		0.5	1.00E+00	1		0.5	7.24E-01
Transport and binding proteins	136	6		1.5	3.00E-01	5		1.0	8.13E-01

Unclassified	303	7	0.8	5.97E-01	11	1.0	8.70E-01
Unknown function	137	4	1.0	1.00E+00	7	1.5	3.36E-01
Viral functions	0	0	0.0	1.00E+00	0	0.0	1.00E+00
Not annotated	138	0	0.0	3.51E-02	1	0.2	9.24E-02
Total	3643	109			128		
True positive rate	-		0.44			0.32	
True negative rate	-		0.836			0.879	
Positive predictive value	-		0.079			0.088	
Negative predictive value	-		0.979			0.973	

*Enriched category. **Under represented category.

Table S4. Transmembrane domain enrichment

Transmembrane domains	Proteins on chip	IgM				IgG			
		Hits		FoldEnrich	p-value	Hits		FoldEnrich	p-value
0	2644	60	**	0.8	8.21E-05	61	**	0.4	1.50E-09
1	492	30	*	2.0	8.67E-05	39	*	1.7	4.44E-07
>1	531	19		1.2	4.08E-01	29	**	0.3	1.47E-02
Total	3667	109				129			
True positive rate	-			0.275				0.302	
True negative rate	-			0.975				0.972	
Positive predictive value	-			0.275				0.302	
Negative predictive value	-			0.975				0.972	

*Enriched category. **Under represented category.

Table S5. Signal peptide enrichment

Signal Peptide	Proteins on chip	IgM				IgG			
		Hits		FoldEnrich	p-value	Hits		FoldEnrich	p-value
Score ≥0.7	562	38	*	2.3	5.00E-07	47	*	2.4	1.92E-09
Score <0.7	3105	72	**	0.8	5.00E-07	82	**	0.8	1.92E-09
Total	3667	109				129			
True positive rate	-			0.345				0.364	
True negative rate	-			0.977				0.974	
Positive predictive value	-			0.345				0.364	
Negative predictive value	-			0.977				0.974	

*Enriched category. **Under represented category.

Table S6. Sub-cellular localization enrichment

Sub-cellular localization	Proteins on chip	IgM				IgG			
		Hits		FoldEnrich	p-value	Hits		FoldEnrich	p-value
Cytoplasmic	1675	38	**	0.8	1.94E-02	35	**	0.6	1.31E-05
Cytoplasmic Membrane	665	24		1.2	3.15E-01	29		1.2	2.00E-01
Extracellular	63	3		1.6	4.37E-01	6	*	2.7	2.22E-02
Outer Membrane	62	6	*	3.2	9.97E-03	9	*	4.1	2.61E-04
Periplasmic	29	0		0	1.00E+00	0		0	6.24E-01
Unknown	1173	38		1.1	6.04E-01	50		1.2	1.02E-01
Total	3667	109				129			
True positive rate	-			0.055				0.116	
True negative rate	-			0.971				0.969	
Positive predictive value	-			0.055				0.12	
Negative predictive value	-			0.971				0.968	

*Enriched category. **Under represented category.

4 CAPÍTULO 2

Artigo intitulado *Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis*, publicado em 31 de janeiro de 2017, na revista *PLoS Neglected Tropical Diseases* (DOI: 10.1371/journal.pntd.0005349).

Neste estudo, o microarranjo fabricado foi utilizado para determinar o perfil de resposta por anticorpos de pacientes com leptospirose branda e grave contra o genoma codificante predito de *L. interrogans* sorovar Copenhageni. Um número limitado de antígenos imunodominantes foi identificado, dos quais 36 foram específicos para pacientes quando comparados a indivíduos saudáveis que residem em área com transmissão endêmica da doença. Dentre os antígenos identificados, 11 foram detectados em pacientes em fase aguda sendo, portanto, antígenos com potencial diagnóstico e 33 foram detectados em pacientes que se recuperaram da doença, sendo possíveis candidatos ao desenvolvimento de uma vacina de subunidade contra leptospirose. Além disso, estes 2 grupos de pacientes apresentaram perfis de anticorpos distintos. Houve um aumento nos níveis de IgG ao longo do tempo no grupo de pacientes com doença grave, perfil observado em mais de 74% dos indivíduos deste grupo, enquanto no grupo com doença branda, este perfil foi evidenciado em apenas 29% dos indivíduos. Em relação a IgM, os níveis permaneceram estáveis em 90% dos pacientes com leptospirose branda contra apenas 51% dos pacientes hospitalizados. Em conjunto, estes resultados sugerem que pacientes com a forma grave da doença estariam tendo uma infecção primária, caracterizada pelo primeiro contato com o patógeno, enquanto pacientes com a doença branda estariam protegidos de manifestações graves pela presença de anticorpos pré-existentes. Um resumo dos antígenos identificados neste estudo é mostrado no Anexo I deste documento.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis

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Abstract

Background

Leptospirosis is an important zoonotic disease worldwide. Humans usually present a mild non-specific febrile illness, but a proportion of them develop more severe outcomes, such as multi-organ failure, lung hemorrhage and death. Such complications are thought to depend on several factors, including the host immunity. Protective immunity is associated with humoral immune response, but little is known about the immune response mounted during naturally-acquired *Leptospira* infection.

Methods and principal findings

Here, we used protein microarray chip to profile the antibody responses of patients with severe and mild leptospirosis against the complete *Leptospira interrogans* serovar Copenhageni predicted ORFeome. We discovered a limited number of immunodominant antigens, with 36 antigens specific to patients, of which 11 were potential serodiagnostic antigens, identified at acute phase, and 33 were potential subunit vaccine targets, detected after recovery. Moreover, we found distinct antibody profiles in patients with different clinical outcomes: in the severe group, overall IgM responses do not change and IgG responses increase over time, while both IgM and IgG responses remain stable in the mild patient group. Analyses of individual patients' responses showed that >74% of patients in the severe group had significant IgG increases over time compared to 29% of patients in the mild group. Additionally, 90% of IgM responses did not change over time in the mild group, compared to ~51% in the severe group.

which is developing products related to the research described in this paper. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict of interest policies. This does not alter our adherence to all PLOS policies on sharing data and materials.

Conclusions

In the present study, we detected antibody profiles associated with disease severity and speculate that patients with mild disease were protected from severe outcomes due to pre-existing antibodies, while patients with severe leptospirosis demonstrated an antibody profile typical of first exposure. Our findings represent a significant advance in the understanding of the humoral immune response to *Leptospira* infection, and we have identified new targets for the development of subunit vaccines and diagnostic tests.

Author summary

Leptospirosis is zoonotic disease of global importance, with over a million cases and nearly 60,000 deaths annually. Symptomatic disease presentation ranges from a mild febrile disease with non-specific symptoms to severe forms, characterized by multi-organ failure, lung hemorrhage, and death. Factors driving severe outcomes remain unclear, but the host immune response likely plays an important role. In the present study, we applied high throughput techniques to identify the antibody profiles of patients with severe and mild leptospirosis. We discovered a limited number of immunodominant antigens, specific to patients. Surprisingly, we found the antibody repertoire varies in patients with different clinical outcomes and hypothesized that patients with mild symptoms were protected from severe disease due to pre-existing antibodies, while the profile of patients with severe outcomes was representative of a first exposure. These findings represent a substantial step forward in the knowledge of the humoral immune response to *Leptospira* infection, and we have identified new targets for vaccine and diagnostic test development.

Introduction

Leptospirosis causes over one million cases and nearly 60,000 deaths annually, with the greatest disease burden in urban slums in tropical and subtropical countries [1–3]. Ten pathogenic *Leptospira* species, over 200 serovars, and a large number of mammalian reservoirs, including rats, have facilitated the emergence of leptospirosis as a major, global public health problem. Humans typically become infected through direct contact with reservoir urine-contaminated soil or water, and develop a broad spectrum of clinical manifestations, including hepato-renal failure and pulmonary hemorrhage syndrome in severe cases, which have high mortality rates [2, 4–6]. The factors contributing to disease severity remain poorly understood, but bacterial virulence, inoculum dose and the host immune response are thought to play important roles in development of severe outcomes [2, 4].

Experimental animal models of *Leptospira* infection have provided a majority of evidence that antibodies play a key role in protection against and clearance of *Leptospira* infection [7–9]. Passive transfer of whole cell leptospiral vaccine and specific anti-leptospiral antibodies (Ligs) are protective against homologous infection in animal models, demonstrating antibodies are sufficient for immunity against experimental homologous infection [10–13]. Additionally, antibodies against LPS are serovar-specific, are correlated with agglutinating antibody titers, and confer limited cross-protection against other serovars [14, 15]. Several studies have shown that leptospirosis patients develop a robust antibody response during infection, especially anti-LPS antibodies, which correspond to the majority of the antibodies produced [12, 16, 17].

The large number of pathogenic *Leptospira* serovars and poor cross-protection observed for anti-LPS antibodies, have made the identification of anti-*Leptospira* protein antibodies a high priority for vaccine and diagnostic test development [18, 19]. In support of this, immunization with an LPS-deficient *Leptospira* strain in experimental animal models conferred cross-protection, implicating anti-protein and other immune responses in protection against infection. [19] Additionally, our group has applied a protein microarray methodology to evaluate the antibody repertoire generated in natural *Leptospira* infection and identified strong antibody responses in healthy exposed individuals as well as several IgG serodiagnostic antigens specific to patients [20, 21].

Analyses of antibody immune responses against infectious agents are essential not only for diagnostic and vaccine development, but also to providing insight in the mechanisms involved in pathogenicity [22]. Protein arrays are an excellent platform that allow for the screening of antibody protein targets in a high-throughput manner, with high sensitivity and high specificity [22–24]. These elements facilitate the assessment of many analytes simultaneously and allow for the identification, quantification and comparison of individual antigenic responses following exposure to microorganisms. Our group has efficiently employed high-density proteome arrays in the characterization of antibody signatures against several infectious agents of human and veterinary importance [25–30], including *Leptospira interrogans* and other spirochetes [21, 31].

In the current study, we used a whole genome proteome microarray approach to describe the first comprehensive profile of the human antibody response to symptomatic *Leptospira* infection. We probed 192 serum samples including patients with different clinical outcomes and healthy controls, and compared their antibody profiles against *L. interrogans* serovar Copenhageni proteins, the serovar associated with >90% of the urban leptospirosis cases in Salvador, Brazil [32, 33]. We identified promising candidates for the development of new diagnostic tests and subunit vaccines and discovered different antibody profiles, which associated with disease severity. Lastly, the antibody kinetics suggest a majority of patients with severe leptospirosis likely have a primary infection, while those with milder disease have evidence of a secondary infection. Our results provide novel insights into the complexity of the immunity in naturally-acquired leptospirosis as well as new diagnostic test candidates.

Methods

Ethics statement

The study protocol was approved by the institutional review board committees of Yale University and Oswaldo Cruz Foundation prior to study initiation. All participants provided written informed consent in their native language prior to sample and data collection. All samples were anonymized before research use.

Study enrollment and sample collection

All 61 patient samples were collected during active surveillance for acute leptospirosis at the Hospital Couto Maia (31 severe group patients) and the São Marcos Emergency Clinic (30 mild group outpatients) in Salvador, Brazil between years 2005–2011. Laboratory confirmation was defined as positive microagglutination test (seroconversion, four-four rise in titer, or single titer $\geq$ 1:800) and/or positive ELISA and/or positive PCR for *Leptospira* DNA as previously described [32]. Serum samples from patients with mild or severe leptospirosis were collected twice: (i) acute sample, collected at patient admittance at the health care unit and (ii) convalescent sample, collected 5–276 days after the first sampling. Controls consisted of (i) 37 sera from healthy *Leptospira*-unexposed (naïve) volunteers from California/US and (ii) 37 sera

from healthy participants enrolled in a cohort study in a high risk urban slum community in Salvador, endemic for leptospirosis.

Leptospira ORF amplification and high throughput cloning

The entire ORFeome of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was amplified by PCR and cloned into pXI vector using a high-throughput PCR recombination cloning method developed by our group [34]. In this strategy, cloned ORFs were expressed with C-terminal hemaglutinin (HA) tag and N-terminal poly-histidine (His) tag. Genes larger than 3kb were cloned as smaller segments as described previously [20, 21] and the *ligA* and *ligB* genes (LIC10465 and LIC10464, respectively) were fragmented according to the repeated Big domains present in the structure of each protein (LigB Repeats 7–12, LigA Repeats 7–13 and LigA/B Repeats 1–6) [35]. After identifying the seroreactive antigens on the microarrays, the inserts in the corresponding plasmids were confirmed by nucleotide sequencing by the Sanger method.

Microarray probing

Microarray fabrication was performed as described previously [20, 21]. Briefly, purified mini-preparations of DNA were used for expression in *E. coli in vitro* based transcription-translation (IVTT) reaction system (RTS Kit, Roche), following the manufacturer´s instructions. Negative control reactions were those performed in the absence of DNA template ("NoDNA" controls). Protease inhibitor mixture (Complete, Roche) and Tween-20 (0.5% v/v final concentration) were added to the reactions, which were then printed onto nitrocellulose coated glass FAST slides using an Omni Grid 100 microarray printer (Genomic Solutions). Multiple negative control reactions and positive control spots of an IgG mix containing mouse, rat and human IgG and IgM (Jackson Immuno Research) were added to the arrays. Protein expression was verified by probing the array with monoclonal anti-polyhistidine (Sigma Aldrich) and anti-hemaglutinin (Roche Applied Science) as previously described [20, 21].

Human sera samples were diluted 1/100 in Protein Array Blocking Buffer (Whatman) supplemented with 10% v/v *E. coli* lysate 10mg/mL (McLab) and incubated 30 min at room temperature (RT) with constant mixing prior to addition to the microarray. Arrays were blocked for 30 min with Protein Array Blocking Buffer and then incubated with diluted samples overnight at 4˚C, with gentle rocking. Washes and incubation with conjugate antibodies were performed as described previously [20, 21]. Slides were scanned in a Perkin Elmer ScanArray confocal laser and intensities were quantified using QuantArray package.

Cloning, expression and purification of recombinant proteins

Selected ORFs were cloned into pET100-TOPO plasmid (Invitrogen) for His-tagged recombinant protein expression in BL21 (DE3) Star *E. coli* cells, according to the manufacturer's recommendations. Recombinant protein expression was performed with EnPresso B system (Biosilta). Briefly, pre-cultured cells were inoculated 1/100 into 3.5 mL of EnPresso B medium supplemented with Ampicilin 100 μg/mL Reagent A 1.5 U/μL and grown shaking (160 rpm) at 30˚C for 16–18 hs in 24-well culture blocks. Expression was induced by the addition of 350 μL of the booster reagent supplemented with 15U/μL Reagent A and 100 mM IPTG, for 24 h at 30˚C under 160 rpm shaking. Cells were then harvested and lysed with 0.05 g of Cellytic Express (Sigma) for each mL of final culture, for 30 min at RT. Lysates were applied to a Ni^{2+}-charged resin (Qiagen) and recombinant proteins were manually purified using 20mM Tris (pH 8.0) buffers with increasing concentrations of Imidazole. Washes varied from 5 mM to 40 mM Imidazole, depending on the protein, and elution was performed with 500 mM or 1M Imidazole. Imidazole

was removed by dialysis (Thermo Scientific dialysis cassettes) and the purified proteins were checked for homogeneity in 12.5% SDS-PAGE. Protein concentration was determined by the BCA method (Thermo Scientific) according to the manufacturer's recommendations.

Multi-antigen print immunoassay

The assay was performed as described previously [23]. Briefly, 100 ng of each purified protein was immobilized on a nitrocellulose membrane strip. A semi-automatic micro-aerolization device was used to generate parallel bands with no visible marks. The membrane was cut into 0.5 cm wide strips perpendicularly to the antigen bands. The strips were blocked for 90 min with 4% reduced-fat bovine milk diluted in PBST (PBS + 0.5% Tween 20) and then incubated for 1 h at RT with individual serum samples diluted 1:200 in PBST 0.25% BSA and 5% v/v *E. coli* lysate 20mg/mL. After 3 washes with PBST, the strips were incubated for 1 hour with alkaline phosphatase–labeled anti-human IgG antibody (Sigma-Aldrich) diluted 1:30.000 in PBST 0.25% BSA. The strips were then washed 3 times with PBST and revealed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Bio-Rad) for 10 min. The reaction was stopped with distilled water. Strips were air-dried and scanned images were converted to gray scale before band intensity quantification with ImageJ software (found at http://rsbweb.nih.gov/ij/).

Protein array data analysis

Array signal intensity was quantified using QuantArray software. Spots intensity raw data were obtained as the mean pixel signal intensity with automatic correction for spot-specific background. Data was normalized by dividing the raw signal for each IVTT protein spot by the median of the sample-specific IVTT control spots (fold-over control [FOC]) and then taking the base-2 logarithm of the ratio (log2 FOC). Conceptually, a normalized signal of 0.0 is equal to control spot signal, and a normalized signal of 1.0 is 2-fold higher than control spot signal.

When evaluating a protein spot as reactive or non-reactive, normalized signals >1.0 were considered reactive. These designations were used to evaluate response frequency and to identify a subset of sero-reactive proteins for further analysis. A given protein on the array was considered sero-reactive if it was reactive in at least 60% of the samples in one or more of the following groups: severe disease, acute sample (n = 30); severe disease, convalescent sample (n = 30); mild disease, acute sample (n = 30); mild disease, convalescent sample (n = 30); endemic controls (n = 30); naïve controls (n = 30). Sero-reactive proteins were identified separately using IgG and IgM responses.

For each sero-reactive protein, sample groups were compared using t-tests [R stats package] and the area under receiver operator characteristic curve (AUC) [R rocr package]. Proteins with t-test p-value < 0.05 after correction for false discovery [36] and AUC > 0.70 were identified as differentially reactive.

Clinical data analysis

Clinical features of the leptospirosis patients participating in this study were described using frequencies and medians with interquartile (IQR) ranges calculated in Excel (Table 1). The Fisher Exact test or the Mann-Whitney test were used to compare clinical presentations of patients with mild or severe disease using GraphPad Prism 5.02 software.

Microarray data accession number

The raw and normalized array data used in this study have been deposited in the Gene Expression Omnibus archive (www.ncbi.nlm.nih.gov/geo/), accession number GSE86630.

Table 1. Clinical characteristics for patients with mild or severe leptospirosis.

CHARACTERISTICS		MILD		SEVERE	
	N	Median (IQR) or N (%)	N	Median (IQR) or N (%)	p-value
Demographics					
Male sex	30	16.0 (53.3)	31	27.0 (87.0)	**0.003**
Age	29	26.5 (17.3–36.8)	30	31.0 (24.5–48.8)	**0.039**
Clinical Presentation[a]					
Days of symptoms[b]					
Acute phase	29	5.5 (3.0–7.8)	30	7.0 (6.0–9.0)	0.085
Convalescent phase	29	44 (22.0–69.5)	30	27.0 (22.5–61.5)	0.681
Hematocrit (%)	12	38.5 (34.5–44.5)	31	34.0 (29.0–37.0)	**0.027**
Platelet count (1000/μL)	30	217.0 (154.3–238.0)	31	73.0 (62.5–177.5)	**0.011**
Laboratory Confirmation					
Agglutinating Antibody Titers					
Acute phase	30	0 (0–175)	31	200 (0–2400)	**0.013**
Convalescent phase	30	300 (0–800)	31	3200 (1600–6400)	**0.0001**
Outcomes					
Respiratory failure[c]	30	0	31	2 (6.5)	0.492
ICU admission	30	0	31	7 (22.5)	**0.011**
Oliguric renal failure[d]	30	0	31	24 (77.4)	**<0.0001**

[a]Values at time of hospital or clinic admission.

[b]Prior to sample collection.

[c]Respiratory failure was defined as presence of pulmonary hemorrhage (>250 ml) or mechanical ventilation.

[d]Oliguric renal failure was defined as oliguria (<500mL urine/day) or anuria (<50ml urine/day) or patient received hemodialysis.

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Results

Patient clinical and laboratory characteristics

To identify antigens associated with symptomatic leptospirosis and severe disease (requiring hospitalization), we enrolled 31 patients hospitalized with suspected leptospirosis, 30 individuals treated at an urgent care facility for suspected leptospirosis, 30 individuals living in the same communities as enrolled patients (hyperendemic controls), and 30 unexposed controls (naïve controls). All patients survived and provided paired acute and convalescent sera samples. Table 1 describes patient characteristics for clinical and biochemical tests performed during hospitalization or outpatient treatment. Hospitalized patients presented with more severe disease: 77.4% had oliguric renal failure, 6.5% had respiratory failure, and 22.5% required ICU admission, while none of these outcomes were observed in outpatients. Additionally, the agglutinating antibody titers for hospitalized patients were significantly higher during acute illness and convalescence compared to patients with mild leptospirosis ($p = 0.011$; $p = <0.0001$). However, while hospitalized patients (severe disease) were older ($p = 0.039$) and predominantly male ($p = 0.03$), there were no significant differences in days of symptoms at acute or convalescent sample collections between patients with mild and severe leptospirosis (acute $p = 0.085$; convalescent $p = 0.681$). Therefore, any differences observed in outcomes were not due to duration of illness or sampling times.

Identification of potential leptospirosis serodiagnostic antigens

In order to determine whether there is an antibody signature specific to symptomatic disease, we probed the protein arrays with a collection of 192 sera samples, including leptospirosis

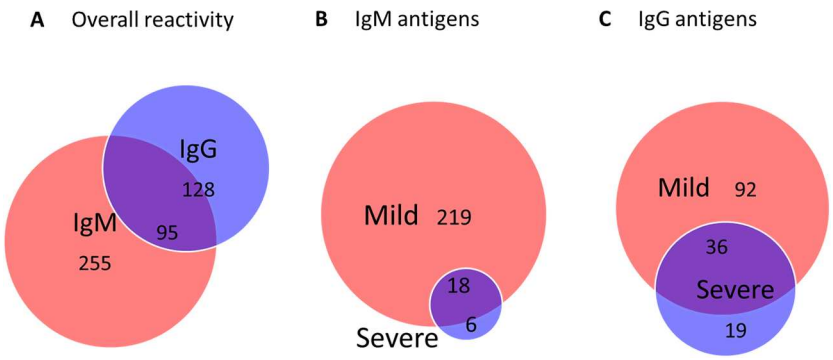

Fig 1. Overall IgM and IgG antibody recognition of leptospiral proteins. (A) Venn diagrams of IgM and IgG leptospiral proteins recognized by humans. Overlap of IgM (B) or IgG (C) sero-reactive antigens identified in patients with mild and severe leptospirosis.

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patients and healthy individuals living in areas with or without endemic transmission of leptospirosis. IgM and IgG probing revealed a set of 478 reactive antigens for both acute and convalescent phases, corresponding to 12.5% of all 3819 proteins and segments included on the arrays. Of these, 255 were specific for IgM, 128 were specific for IgG and 95 were recognized by both antibodies (Fig 1A). Interestingly, we detected a majority of the IgM and IgG antigens in patients with mild disease (Fig 1B and 1C). To identify antigens specific to patients with confirmed leptospirosis (serodiagnostic antigens), we then compared antigens from the sera of patients with those from healthy individuals and found 36 antigens with significantly higher IgG reactivity in leptospirosis patients than in healthy volunteers from United States or healthy individuals living in a highly endemic area in Brazil. Of these, 12 (33%) were identified during acute leptospirosis (S2 Table) and 33 (92%) during convalescence (S3 Table).

Early antigen detection during infection is critical for the development of a new diagnostic test for leptospirosis. Therefore, we first focused on serodiagnostic antigens identified during acute phase in patients with mild or severe disease. Surprisingly, we found only a limited subset of all the seroreactive antigens were significantly recognized by IgGs in patients relative to endemic and naïve control volunteers: 11 of the 128 in the mild patient group and 28 of the 55 in the severe group (Fig 2A). Of these only 5 of the 11 and 9 of the 28 were present during acute illnesss. For the mild group, the Lig proteins were the antigens with highest accuracy, especially LigA/B 1–6, with 90% sensitivity, 86% specificity and AUC of 0.916. To determine whether we could increase both sensitivity and specificity by combining the antigens, we constructed Receiver Operating Characteristic (ROC) curves for combinations of the 5 antigens to assess antigens diagnostic performance (Fig 2A). We found that combining the top two antigens LigA/B 1–6 and LigA 8–13 yielded slightly higher sensitivity (86%) and specificity (91%) than the other combinations (Fig 2A). We performed similar analyses for the 9 antigens specific to the severe group. Again, the best diagnostic accuracy was achieved with LigA/B 1–6 (AUC = 0.935, 87% sensitivity, 100% specificity) followed by LIC20276 (AUC = 0.901, 84% sensitivity, 92% specificity). When we combined both antigens, sensitivity reached 94%, and specificity was 100% (Fig 2B). For the remaining antigens, sensitivity ranged from 77% to 90% and specificity ranged from 77% to 92%. Again, other combinations did not yield better combined sensitivity and specificity (Fig 2B). Our results indicate that we have identified candidates for new leptospirosis diagnostic tests and have discovered that there may be a limited dominant antigen antibody response to *Leptospira* infection.

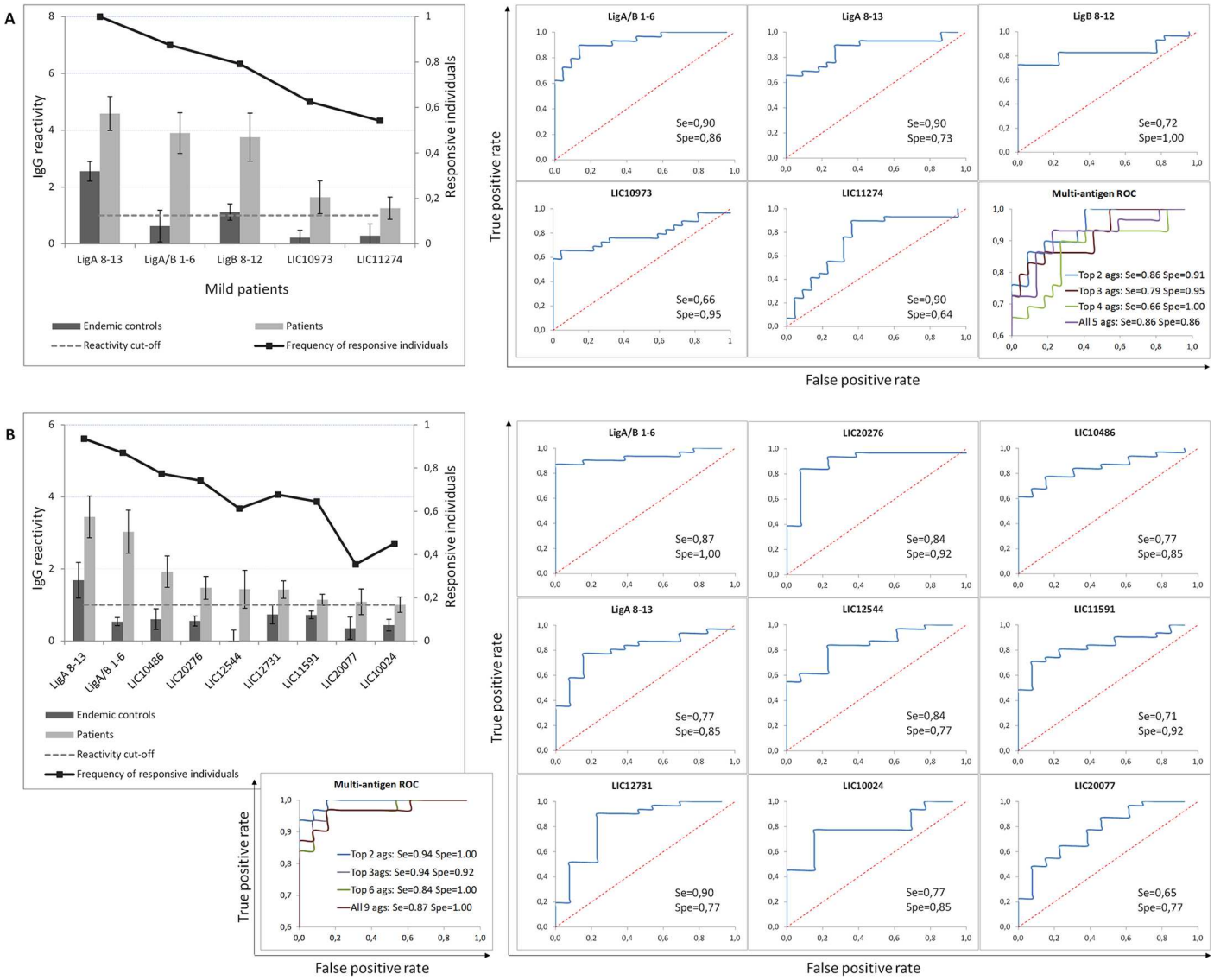

Fig 2. Serodiagnostic antigens identified for patients with mild and severe leptospirosis. Histograms plot the average normalized intensity (Y axis) of each antigen (X axis) for hyperendemic controls (dark gray bars) and patients with mild (A) or severe (B) disease (light gray bars), with the frequency of responsive individuals (black line, secondary axis). Error bars indicate S.E. Single or multi-antigens ROC curves of the identified serodiagnostic antigens for mild (A) or severe (B) groups are shown with sensitivity and specificity rates.

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Identification of potential subunit vaccine candidates in recovered leptospirosis patients

We analyzed the responses from convalescent sera to determine whether there were major shifts in antibody responses to specific antigens with time. Patients recovering from mild disease had significantly higher IgG titers for 10 antigens compared to endemic controls, while the number of antigens nearly tripled for patients with severe clinical presentation (S3 Table). Antigens identified at convalescent phase accounted for ~92% of all diagnostic antigens (33 in 36 total IgG antigens) and LigA/B 1–6 and LigB 8–12 were the antigens with best diagnostic performance for patients with severe and mild disease, respectively. While these antigens do

not have diagnostic potential, they do represent possible subunit vaccine candidates as robust antibody responses were generated over the duration of illness.

Protein microarray validation by MAPIA

To confirm the diagnostic and subunit vaccine potential of the sero-reactive antigens detected on the microarray chips, we purified six proteins from *E. coli* BL21 *in vitro* (Fig 3B), and printed onto nitrocellulose membranes. We probed the immunostrips with serum from 8 endemic controls and 20 acute-phase patients, of which 10 had mild disease and 10 had severe disease. Serum from leptospirosis patients showed greater reactivity than serum from controls, especially serum from severe patients at convalescent phase (Fig 3A). To assess the ability of these six antigens to distinguish between patients and controls, a multi-antigen ROC curve was generated (Fig 3C), and demonstrated that the six selected antigens yielded a specificity of 100% and a sensitivity of 60% for acute mild group and 90% for the remaining groups.

Distinct antibody profiles associated with disease presentation

As there is limited knowledge of the factors contributing to leptospirosis severe disease outcomes, we compared the antibody kinetics of patients to determine whether there are differences in antibody responses based on disease severity. We first compared the global IgG and IgM reactivities against all 478 reactive antigens identified in the microarrays by comparing the summed average signal intensities for each antigen during acute illness with that at convalescence. We detected a trending increase in IgG reactivity in patients with severe leptospirosis,

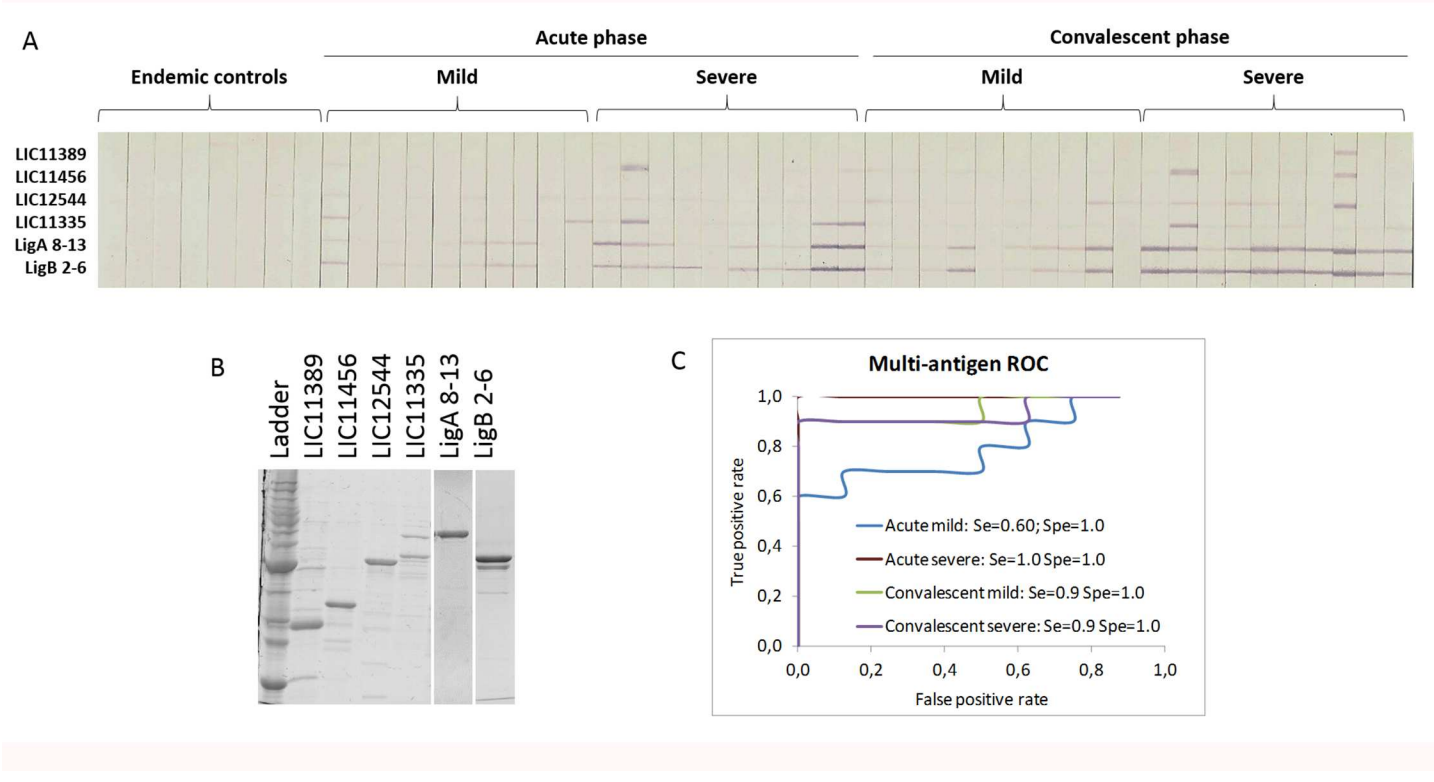

Fig 3. Validation of microarray results by MAPIA. (A) MAPIA strips probed for specific IgG in leptospirosis patients and endemic controls. Strips are grouped by disease severity, in acute and convalescent phases. (B) SDS PAGE of the 6 purified recombinant proteins that were applied to MAPIA strips. (C) ROC curves of the combination of all 6 antigens are shown for mild and severe groups at both acute and convalescent phases.

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which reached statistical significance when we analyzed the signals from the 36 patient-specific antigens ($p<0.05$) (S2 Fig). We did not observe this trend in patients with mild disease. For IgM-specific antigens, we observed no significant differences for either patient group or antigen set (S2 Fig). Thus, we identified significant IgG responses increases only in the severe patient group over time.

To understand the differences in antibody kinetics in patients in more detail, we next compared the antibody responses to the 36 differentially reactive antigens at the acute and convalescent time points for each individual by two way t-test. Based on the results of each t-test the individuals were categorized as: (i) increasing, when average response to the 36 differentially reactive antigens was higher at convalescent time point than acute, and p-value < 0.05), (ii) no change (p-value > 0.05) or (iii) decreasing, when average response to the 36 differentially reactive antigens was lower at convalescent time point than acute, and p-value < 0.05. This comparison yielded vastly different profiles for patients with mild disease and severe disease. When analyzing IgG responses, we categorized 74.4% of patients with in the severe group as "increasing" versus only 29.6% of patients in the mild group (Fig 4A). When analyzing IgM responses, we categorized 32.3% of patients in the severe group as "increasing" versus only 3.3% in the mild group (Fig 4B). Additionally, 90.0% of IgM responses did not change over time in the mild group, compared to 51.6% in the severe group. Altogether, these data clearly demonstrate that leptospirosis patients with different clinical presentations generate distinct antibody profiles.

In our kinetic antibody analyses, we enrolled five patients with mild leptospirosis, which had antibody profiles that resembled those of patients with severe leptospirosis: all had increases in IgG levels over time for 10 antigens (Fig 4C and S2). Though these five patients clearly developed an antibody response more representative of patients with severe disease (S3 Fig), including a higher convalescent agglutinating antibody titer (400–12800), they did not present with any severe clinical outcomes we measured. All other clinical and laboratory features were similar to the 25 patients with mild leptospirosis (S4 Table).

Discussion

Leptospirosis is a disease with a broad spectrum of clinical manifestations ranging from asymptomatic and nonspecific acute febrile illnesses to life-threatening renal failure or pulmonary hemorrhage syndrome [2, 37]. Over a million cases of severe leptospirosis occur every year. This figure represents only a faction (potentially 5–15%) of the total mild leptospirosis cases, which usually are not identified by surveillance systems. The mechanisms involved in poor disease progression remain poorly defined, but pathogen related and host factors likely contribute to this heterogeneity [2, 4]. Here, we identified 12 specific IgG antigens that differentiate acute symptomatic disease from uninfected individuals in endemic regions and therefore represent promising diagnostic candidates for an early laboratory test for the diagnosis of leptospirosis. We also identified patient-specific antigens during convalescence, which are putative subunit vaccine candidates. Lastly, we showed that patients with different clinical presentations generate distinct antibody kinetic profiles, and we hypothesize that since antibodies are protective, disease severity and the antibody signatures may indicate primary and secondary infections.

We identified 12 IgG serodiganostic antigens for acute leptospirosis. Among them are the well-known sero-reactive proteins LigA/B 1–6, LigA 8–13, LigB 8–12 and LIC10973 (OmpL1). Several published studies used the Ligs as diagnostic markers for leptospirosis [35, 38–41] as well as OmpL1, especially in combination with LipL21, LipL32 or LipL41 [42]. Our group has previously identified LIC10486 (hypothetical protein) and LIC12544 (DNA binding protein) using the protein microarray platform [21]. The remaining 6 proteins LIC10024 (adenylate/

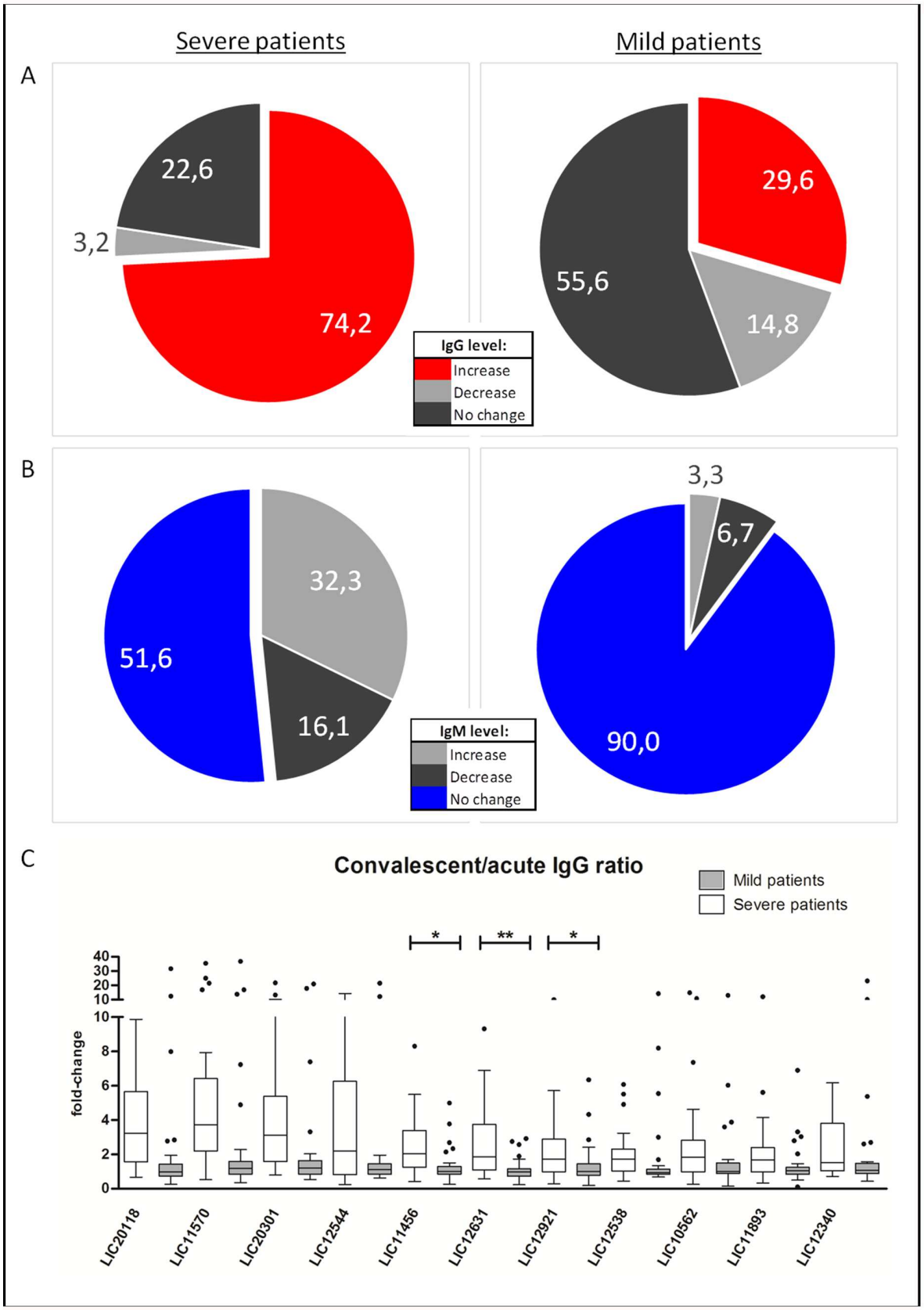
Severe patients
Mild patients
A
22,6
3,2
74,2
29,6
55,6
14,8
IgG level:
Increase
Decrease
No change
B
32,3
51,6
16,1
3,3
6,7
90,0
IgM level:
Increase
Decrease
No change
C
Convalescent/acute IgG ratio
Mild patients
Severe patients
*
**
*
fold-change
40
30
20
10
10
8
6
4
2
0
LIC20118
LIC11570
LIC20301
LIC12544
LIC11456
LIC12631
LIC12921
LIC12538
LIC10562
LIC11893
LIC12340

Fig 4. IgM and IgG antibody kinetics in patients with mild and severe leptospirosis. Percentage of patients that showed increase, decrease or unchanged IgG (A) and IgM (B) levels from acute to convalescent phases. Patients with severe disease are shown on the left and patients with mild form, on the right. (C) Boxplot shows the IgG fold-change (y-axis) of mild (dark gray) and severe (light gray) groups for each of the antigens in the x-axis. Significant differences are marked with star (*p<0.01; **p<0.001).

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guanylate cyclase), LIC11591 (exodeoxyribonuclease VII large subunit), LIC20077 (polysaccharide deacetylase) and the hypothetical proteins LIC11274, LIC20276 and LIC12731 are promising newly identified serodiagnostic antigens, especially LIC20276, which improved diagnostic performance for severe disease in combination with LigA/B 1–6. Interestingly, patients showed antibody reactivity against several proteins annotated as hypothetical proteins, not only at acute disease, but also during convalescence. These results indicate that even though these proteins have not been assigned any function, they are indeed expressed by the bacteria and might play an important role in host infection. Further studies should be done in order to evaluate these antigens performance in different diagnostic platforms, such as ELISA and rapid tests. For diagnostic purposes, a complete validation study needs to be performed, including the probing of a more extensive sample collection, comprising more leptospirosis patients as well as healthy controls and patients with other febrile illness, such as dengue, sifilis and hepatitis A.

The results presented here are consistent with our previous findings [21]. We detected 13 of the 24 IgG antigens previously found in hospitalized patients, strengthening the diagnostic potential of those antigens and validating the protein microarray antigen discovery platform. The inclusion of 39% of *L. interrogans* predicted ORFeome, however, did not provide significant advantage in diagnostic antigen discovery, since only 3 out of the 1489 proteins and segments added to the microarray were serodiagnostic, indicating that the algorithm used by our group to select the proteins included in the partial microarray was effective. Indeed, 32 out of the 36 diagnostic antigens identified here fall in at least one of the enrichment categories described by our group for antibody recognition [20, 43, 44].

Leptospirosis patients and healthy controls reacted against 12% of the *L. interrogans* predicted ORFeome. The majority of the imunodominant antigens were IgM specific, which corresponded to >50% of the sero-reactive proteins. The high number of IgM antigens may reflect the broad and low-affinity antigen-antibody interaction typical of IgM antibodies [45, 46]. These features usually make IgM a hard indicator of reliable diagnostic tests and might have hindered the identification of IgM diagnostic targets, as they usually account for lower specificity in IgM-based serological tests and high background reactivity in negative samples [45]. Here, we had great success in detecting IgG antigens with potential use as diagnostic or vaccine targets, but further studies are needed to identify IgM antigens.

In our previous work, we have shown that healthy individuals who live in areas with endemic transmission of leptospirosis have a background IgG reactivity against leptospiral protein antigens, possibly due to the constant exposure to the pathogen [21]. As it is well known that antibodies are one of the main immune mechanisms in naturally-acquired leptospirosis [16], the presence of high IgG levels in such individuals suggests that those antibodies might play an important role in protection against the development of clinical leptospirosis. Despite this background IgG reactivity, we were able to identify antigens for which IgG levels were even higher among hospitalized leptospirosis patients, especially at the patient's convalescent sample [21]. Indeed, most of the 36 serodiagnostic antigens identified in the present study were detected in the convalescent sample of patients with severe disease. A considerably smaller number of antigens was detected in patients with the mild form, suggesting that their IgG antibody response is more similar to healthy individuals living in the same area.

The distinct antibody profiles associated with each group were not due to differences in days of symptoms. We hypothesize that patients with mild leptospirosis had a background IgG reactivity that protected them from severe clinical manifestations while the lack of such IgG response might have favored the development of severe outcomes in hospitalized patients. In general, the first contact with an infectious agent is serologically characterized by a gradual increase in IgM, with a peak on days 7–10 after pathogen exposure, followed by an increase in IgG on days 10–14. In a secondary infection, however, a robust IgG response is rapidly mounted as a consequence of the activation of memory B cells generated during the primary infection [47–49]. In the light of this, the fact that patients with mild leptospirosis maintained their IgG levels at acute serum sample, collected approximately 5 days after the onset of symptoms, and at convalescent sample, collected at least 13 days later, suggests that they mounted an anamnestic response due to a secondary leptospiral infection. In contrast, patients with the severe form showed an antibody response typical of a primary infection, with an increase in IgG levels from acute to convalescent phases.

Our results indicate that the presence of antibodies anti-leptospiral proteins may be protective against clinical severe leptospirosis and that patients with mild disease might have had previous leptospiral infection(s). However, numerous aspects can affect the host immune response against an infectious agent, including the inoculum size. Patients with severe clinical presentations might have been infected with a higher bacterial load than patients who presented the mild form, developing thereby a more intense immune response. In addition, we can't affirm that any of the patients enrolled in the present study had never been exposed to leptospira before since leptospirosis is highly endemic in their community. Nonetheless, there is a need of studies of this kind to help elucidate the immune response associated with naturally-acquired leptospirosis and we believe our work brings relevant information to the field.

Supporting information

S1 Fig. Representative agarose gels and microarray images. (A) Representative agarose gels of PCR amplifications and plasmid mini-preparations. All PCR amplicons and plasmid mini-preparations were verified in agarose gels before microarray production. (B) Two subarrays showing His (left) and HA (right) probing for protein expression evaluation. Each microarray chip contained 16 subarrays. Highlighted spots correspond to IVTT control reactions (NoDNA, red boxes), IgGmix (blue) and human IgM (green).
(TIF)

S2 Fig. Patients cumulative IgM nd IgG reactivity against sero-reactive antigens. Summed average signal intensity is shown (y-axis) as the number of antigens (x-axis) increases. Cumulative reactivity is shown for patients with (left) and mild (right) illness against all 478 reactive antigens (up) and the 36 serodiagnostic antigens.
(TIF)

S3 Fig. Heatmap of the antibody fold-change from acute to convalescent illness for 10 immunodominant antigens. Fold-change is represented according to the colorized scale with red strongest, black in-between and green weakest. Antigens are in rows; patient samples are in columns, grouped by clinical presentation and sorted from left to right by increasing average antigen intensity within each group. IgM fold-change is shown on the left; IgG fold-change is shown on the right. The five outliers in the mild group are highlighted with star (*).
(TIF)

S1 Table. Proteins not represented on the microarrays.
(DOCX)

S2 Table. Acute phase serodiagnostic antigens identified in patients with mild and severe leptospirosis.
(DOCX)

S3 Table. Convalescent phase serodiagnostic antigens identified in patients with mild and severe leptospirosis.
(DOCX)

S4 Table. Clinical characteristics for mild leptospirosis patients with distinct antibody kinetics.
(DOCX)

S1 Results.
(DOCX)

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Visualization: CLA.

Writing – original draft: CLA JCL.

Writing – review & editing: CLA JCL AR FC PLF.

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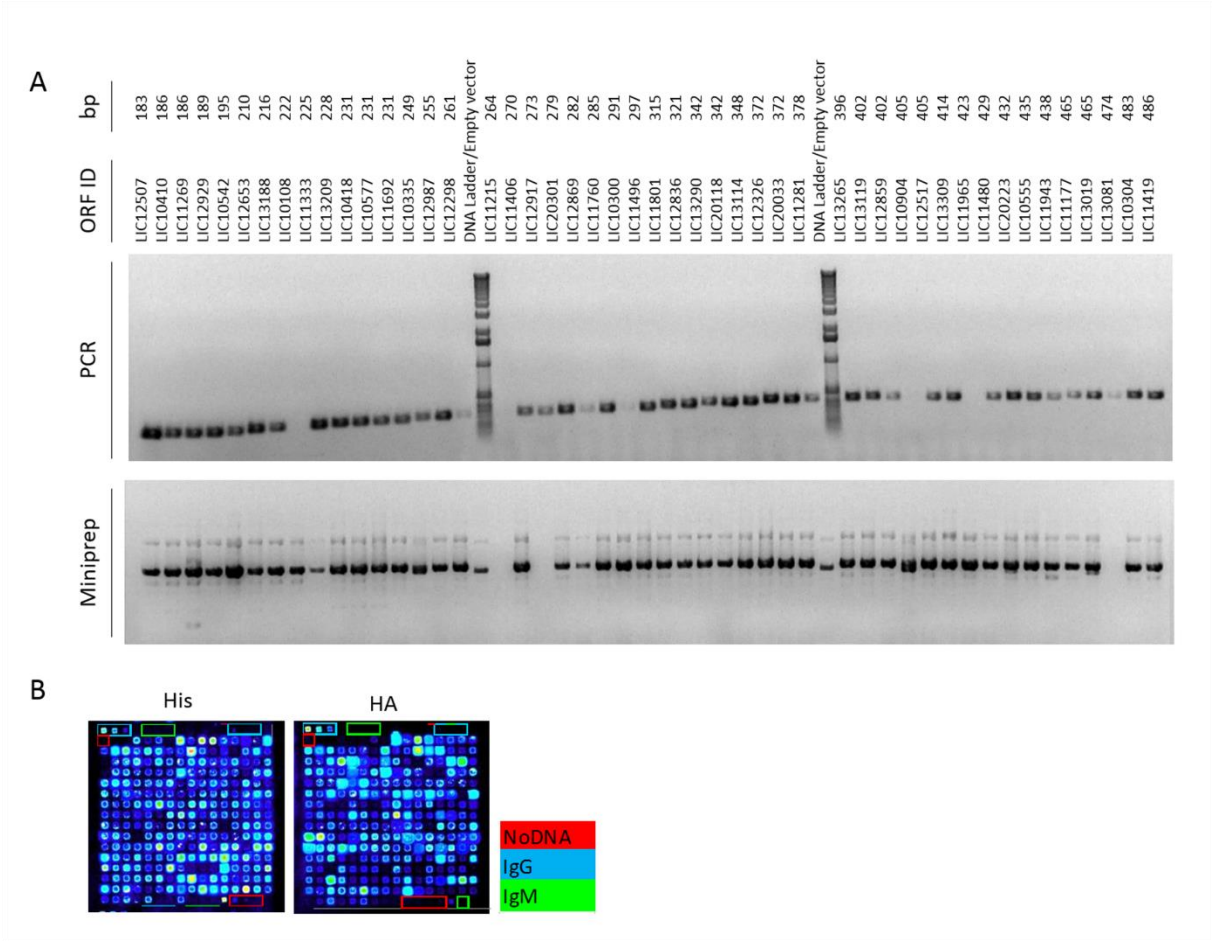
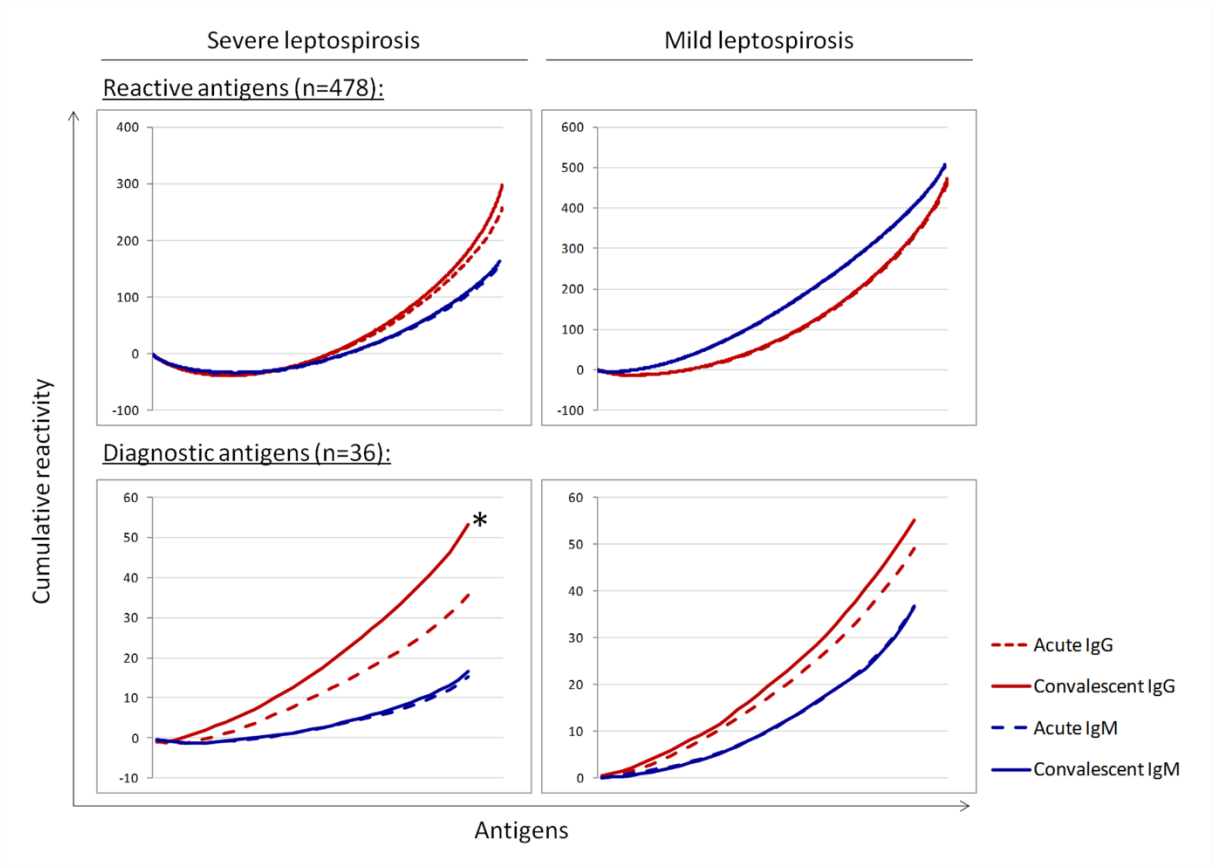
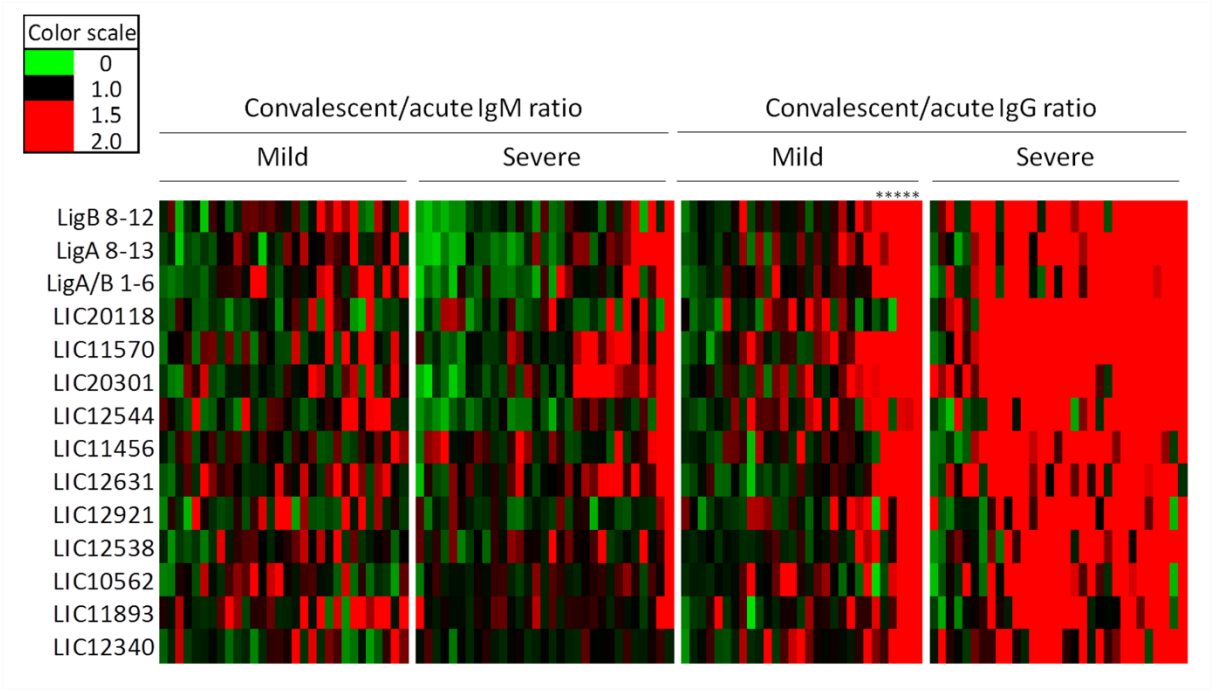
Supplementary information



S1 Figure: Representative agarose gels and microarray images. (A) Representative agarose gels of PCR amplifications and plasmid mini-preparations. All PCR amplicons and plasmid mini-preparations were verified in agarose gels before microarray production. (B) Two subarrays showing His (left) and HA (right) probing for protein expression evaluation. Each microarray chip contained 16 subarrays. Highlighted spots correspond to IVTT control reactions (NoDNA, red boxes), IgGmix (blue) and human IgM (green).



S2 Figure: Patients cumulative IgM nd IgG reactivity against sero-reactive antigens. Summed average signal intensity is shown (y-axis) as the number of antigens (x-axis) increases. Cumulative reactivity is shown for patients with (left) and mild (right) illness against all 478 reactive antigens (up) and the 36 serodiagnostic antigens.



S3 Figure: Heatmap of the antibody fold-change from acute to convalescent illness for 10 immunodominant antigens. Fold-change is represented according to the colorized scale with red strongest, black in-between and green weakest. Antigens are in rows; patient samples are in columns, grouped by clinical presentation and sorted from left to right by increasing average antigen intensity within each group. IgM fold-change is shown on the left; IgG fold-change is shown on the right. The five outliers in the mild group are highlighted with star (*).

Supplementary tables

S1 Table: Proteins not represented on the microarrays.

Locustag	Accession	Product Name
LIC10889	YP_000866.1	TonB
LIC11008	YP_000981.1	hypothetical protein LIC11008
LIC11109	YP_001079.1	hypothetical protein LIC11109
LIC11352	YP_001316.1	LipL32
LIC11364	YP_001328.1	short-chain dehydrogenase
LIC11885	YP_001834.1	putative lipoprotein
LIC12227	YP_002162.1	hypothetical protein LIC12227
LIC12322	YP_002256.1	glutaconate CoA transferase-like protein
LIC13131	YP_003039.1	hypothetical protein LIC13131
LIC20250	YP_003634.1	peptidoglycan-associated cytoplasmic membrane protein
LIC20254	YP_003638.1	response regulator
LIC10415	YP_000401.1	hypothetical protein LIC10415
LIC20044	YP_003436.1	heat shock protein 90
LIC13136	YP_003044.1	acyl-CoA dehydrogenase
LIC11791	YP_001743.1	ATP-dependent DNA helicase
LIC10772	YP_000752.1	hypothetical protein LIC10772
LIC10037	YP_000037.1	GMC oxidoreductase
LIC11499	YP_001460.1	hypothetical protein LIC11499
LIC11898	YP_001847.1	pyruvate dehydrogenase subunit beta
LIC10958	YP_000932.1	alcohol dehydrogenase
LIC11620	YP_001578.1	DNA repair protein
LIC11707	YP_001659.1	fructose-1,6-bisphosphatase
LIC10838	YP_000815.1	glyoxalase
LIC12414	YP_002345.1	hypothetical protein LIC12414
LIC12958	YP_002871.1	hypothetical protein LIC12958
LIC11423	YP_001385.1	glycine rich RNA-binding protein
LIC12744	YP_002664.1	NADH dehydrogenase subunit I D
LIC12251	YP_002186.1	L-aspartate oxidase
LIC13194	YP_003102.1	cholesterol oxidase
LIC11500	YP_001461.1	methionine aminopeptidase
LIC10712	YP_000696.1	hypothetical protein LIC10712
LIC11851	YP_001800.1	cytoplasmic membrane protein
LIC12679	YP_002601.1	thiamine biosynthesis protein ThiC
LIC11854	YP_001803.1	putative methyltransferase
LIC11989	YP_001928.1	hypothetical protein LIC11989
LIC10163	YP_000155.1	hypothetical protein LIC10163
LIC10241	YP_000232.1	hypothetical protein LIC10241
LIC11514	YP_001473.1	hypothetical protein LIC11514
LIC12331	YP_002263.1	hypothetical protein LIC12331
LIC12675	YP_002597.1	hypothetical protein LIC12675

LIC13261	YP_003168.1	hypothetical protein LIC13261
LIC20156	YP_003547.1	hypothetical protein LIC20156
LIC11134	YP_001104.2	histone deacetylase
LIC10434	YP_000419.1	hypothetical protein LIC10434
LIC10723	YP_000707.1	flagellar hook-associated protein FliD
LIC11062	YP_001032.1	hypothetical protein LIC11062
LIC20192	YP_003583.1	hypothetical protein LIC20192
LIC11433	YP_001395.1	histidine kinase sensor protein
LIC13032	YP_002945.1	hypothetical protein LIC13032
LIC12646	YP_002569.1	membrane carboxypeptidase
LIC10298	YP_000288.1	flagellar basal body rod protein FlgC
LIC12993	YP_002906.1	hypothetical protein LIC12993
LIC10021	YP_000021.1	putative lipoprotein
LIC10137	YP_000133.1	[protein-PII] uridylyltransferase
LIC10417	YP_000403.1	hypothetical protein LIC10417
LIC10774	YP_000754.1	putative lipoprotein
LIC10784	YP_000763.1	transposase, IS1501
LIC10785	YP_000764.1	transposase, IS1501
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LIC11033	YP_001003.1	hypothetical protein LIC11033
LIC11225	YP_001194.1	hypothetical protein LIC11225
LIC11242	YP_001211.1	F0F1 ATP synthase subunit gamma
LIC11305	YP_001271.1	hypothetical protein LIC11305
LIC11306	YP_001272.1	hypothetical protein LIC11306
LIC11321	YP_001287.1	hypothetical protein LIC11321
LIC11382	YP_001346.1	hypothetical protein LIC11382
LIC11432	YP_001394.1	histidine kinase response regulator hybrid protein
LIC11587	YP_001546.1	ribonucleotide-diphosphate reductase subunit alpha
LIC11690	YP_001642.1	hypothetical protein LIC11690
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LIC11995	YP_001934.1	hypothetical protein LIC11995
LIC12111	YP_002045.2	30S ribosomal protein S18
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LIC12249	YP_002184.1	hypothetical protein LIC12249
LIC12385	YP_002317.1	hypothetical protein LIC12385
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LIC12558	YP_002485.1	hypothetical protein LIC12558
LIC12637	YP_002560.1	50S ribosomal protein L31
LIC12839	YP_002755.1	aspartate carbamoyltransferase catalytic subunit
LIC12855	YP_002771.1	50S ribosomal protein L30
LIC12860	YP_002776.1	30S ribosomal protein S14
LIC12865	YP_002781.1	50S ribosomal protein L29
LIC13013	YP_002926.1	hypothetical protein LIC13013
LIC13034	YP_002947.1	hypothetical protein LIC13034
LIC13109	YP_003018.1	hypothetical protein LIC13109
LIC13163	YP_003071.1	3-methyl-2-oxobutanoate hydroxymethyltransferase
LIC13182	YP_003090.1	glycerophosphoryl diester phosphodiesterase

LIC13221	YP_003129.1	Fe-S oxidoreductase
LIC13391	YP_003294.1	hypothetical protein LIC13391
LIC13422	YP_003325.1	hydrogenase subunit
LIC14000	YP_003858659.1	hypothetical protein LIC14000
LIC20065	YP_003457.1	acyl carrier protein
LIC20263	YP_003647.1	hypothetical protein LIC20263
LIC20300	YP_003858670.1	hypothetical protein LIC20300
LIC12693	YP_002614.1	hypothetical protein LIC12693
LIC13301	YP_003206.1	acetyl-CoA acetyltransferase
LIC10583	YP_000567.1	acyl-CoA dehydrogenase
LIC13009	YP_002922.1	acyl-CoA dehydrogenase
LIC10445	YP_000430.1	glucosamine--fructose-6-phosphate aminotransferase
LIC11571	YP_001530.1	general secretory pathway protein E
LIC20175	YP_003566.1	dnaK suppressor
LIC12026	YP_001964.1	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methylene-tetrahydrofolate cyclohydrolase
LIC12535	YP_002463.1	riboflavin synthase subunit alpha
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LIC13020	YP_002933.2	hypothetical protein LIC13020
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LIC20259	YP_003643.1	hypothetical protein LIC20259
LIC10485	YP_000469.1	hypothetical protein LIC10485
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LIC12700	YP_002621.1	metalloprotease
LIC12163	YP_002098.1	hypothetical protein LIC12163
LIC12785	YP_002702.1	ribosomal RNA large subunit methyltransferase N
LIC12302	YP_002236.1	putative DNA-binding/iron metalloprotein/AP endonuclease
LIC13280	YP_003187.1	MaoC family protein
LIC10668	YP_000652.1	Mrr
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LIC12428	YP_002359.1	ABC transporter ATP-binding protein
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LIC11502	YP_001463.1	hypothetical protein LIC11502
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LIC11827	YP_001778.1	lipopolysaccharide biosynthesis glycosyltransferase
LIC11867	YP_001816.1	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
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LIC20180	YP_003571.1	response regulator
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LIC20209	YP_003598.1	methylmalonyl-CoA mutase
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LIC12551	YP_002478.1	acyl-CoA dehydrogenase
LIC12027	YP_001965.1	hypothetical protein LIC12027
LIC10538	YP_000522.1	ABC transporter ATP-binding protein
LIC13219	YP_003127.2	phenylalanyl-tRNA synthetase subunit beta
LIC12792	YP_002709.1	pantothenate kinase
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LIC10344	YP_000334.1	anti-sigma factor antagonist
LIC11741	YP_001693.1	hypothetical protein LIC11741
LIC10222	YP_000213.1	DNA polymerase III subunit alpha
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LIC20222	YP_003611.1	hypothetical protein LIC20222
LIC10950	YP_000924.1	sigma factor regulatory protein
LIC13218	YP_003126.1	carbonic anhydrase/acetyltransferase
LIC10161	YP_000153.1	hypothetical protein LIC10161
LIC10227	YP_000218.1	hypothetical protein LIC10227
LIC13118	YP_003027.1	amino-sugar biosynthesis protein
LIC10529	YP_000513.1	hypothetical protein LIC10529
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LIC10563	YP_000547.1	SenC
LIC10639	YP_000623.1	hypothetical protein LIC10639
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LIC10917	YP_000892.1	hypothetical protein LIC10917
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LIC11002	YP_000975.1	IS1533 transposase
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LIC11269	YP_001238.1	hypothetical protein LIC11269
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LIC11323	YP_001289.1	methyltransferase DNA modification enzyme
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LIC11497	YP_001458.1	methyltransferase
LIC11717	YP_001669.1	excinuclease ABC subunit A
LIC11746	YP_001698.1	N-acetyl-gamma-glutamyl-phosphate reductase
LIC11774	YP_001726.1	DNA mismatch repair protein MutS
LIC12335	YP_002267.1	serine hydroxymethyltransferase
LIC12370	YP_002302.1	hypothetical protein LIC12370
LIC12386	YP_002318.1	drug/metabolite exporter
LIC12406	YP_002338.1	hypothetical protein LIC12406
LIC12417	YP_002348.1	GTP-binding protein EngA
LIC12461	YP_002389.1	50S ribosomal protein L20
LIC12650	YP_002573.1	hypothetical protein LIC12650
LIC12670	YP_002592.1	adenylate/guanylate cyclase
LIC12688	YP_002609.1	exodeoxyribonuclease v subunit gamma
LIC12697	YP_002618.1	hypothetical protein LIC12697
LIC12750	YP_002670.1	NADH dehydrogenase I subunit L
LIC12752	YP_002672.1	NADH dehydrogenase I subunit N
LIC12753	YP_002673.1	hypothetical protein LIC12753
LIC12784	YP_002701.1	putative lipoprotein
LIC12825	YP_002741.1	hypothetical protein LIC12825
LIC12869	YP_002785.1	30S ribosomal protein S19
LIC13081	YP_002993.1	hypothetical protein LIC13081
LIC13129	YP_003037.1	methionyl-tRNA synthetase
LIC13291	YP_003198.1	ankyrin domain-containing protein
LIC13304	YP_003209.1	hypothetical protein LIC13304
LIC13317	YP_003222.1	hypothetical protein LIC13317
LIC13320	YP_003225.1	thermolysin precursor
LIC13322	YP_003227.1	thermolysin precursor
LIC13430	YP_003333.1	hypothetical protein LIC13430
LIC13468	YP_003370.1	hypothetical protein LIC13468
LIC14005	YP_003858660.1	hypothetical protein LIC14005
LIC20255	YP_003639.1	hypothetical protein LIC20255
LIC10382	YP_000368.1	acyl-CoA dehydrogenase
LIC10543	YP_000527.1	acyl-CoA dehydrogenase
LIC11173	YP_001143.1	hypothetical protein LIC11173
LIC12505	YP_002433.1	response regulator
LIC12762	YP_002682.1	hypothetical protein LIC12762
LIC20198	YP_003587.1	hypothetical protein LIC20198
LIC11257	YP_001226.1	exopolyphosphatase

LIC12350	YP_002282.1	glycyl-tRNA synthetase
LIC13448	YP_003351.1	carbon storage regulator
LIC10174	YP_000166.1	hypothetical protein LIC10174
LIC10896	YP_000873.1	TonB-dependent outer membrane receptor
LIC20009	YP_003401.1	porphobilinogen deaminase
LIC12701	YP_002622.1	polynucleotide phosphorylase/polyadenylase
LIC12421	YP_002352.1	hypothetical protein LIC12421
LIC12205	YP_002140.1	GDP-l-fucose synthetase
LIC11483	YP_001444.1	hypothetical protein LIC11483
LIC20088	YP_003480.1	diphosphate--fructose-6-phosphate 1-phosphotransferase
LIC10151	YP_000145.1	hypothetical protein LIC10151
LIC12536	YP_002464.1	hypothetical protein LIC12536
LIC10350	YP_000339.1	hypothetical protein LIC10350
LIC12413	YP_002344.1	hypothetical protein LIC12413
LIC20265	YP_003649.1	hypothetical protein LIC20265
LIC13308	YP_003213.1	hypothetical protein LIC13308
LIC10322	YP_000312.1	hypothetical protein LIC10322
LIC10457	YP_000441.1	hypothetical protein LIC10457
LIC10556	YP_000540.1	hypothetical protein LIC10556
LIC11034	YP_001004.1	hypothetical protein LIC11034
LIC11246	YP_001215.1	hypothetical protein LIC11246
LIC11248	YP_001217.1	alginate o-acetyltransferase
LIC11453	YP_001414.1	histidinol dehydrogenase
LIC11460	YP_001421.1	aspartyl/glutamyl-tRNA amidotransferase subunit C
LIC11558	YP_001517.1	tRNA (guanine-N(1)-)-methyltransferase/unknown domain fusion protein
LIC11647	YP_001605.1	pseudouridylate synthase
LIC11655	YP_001611.1	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
LIC11869	YP_001818.1	S-adenosyl-methyltransferase
LIC12025	YP_001963.1	asparaginyl-tRNA synthetase
LIC12562	YP_002489.1	putative ring hydroxylating dioxygenase alpha-subunit
LIC13404	YP_003307.1	acetyl-CoA synthetase
LIC20055	YP_003447.1	hypothetical protein LIC20055
LIC20110	YP_003502.1	hypothetical protein LIC20110
LIC10464	YP_000448.1	Ig-like repeat-containing protein
LIC10465	YP_000449.1	Ig-like repeat-containing protein
LIC11739	YP_001691.1	hypothetical protein LIC11739
LIC11026	YP_000996.1	hypothetical protein LIC11026
LIC12602	YP_002525.1	hypothetical protein LIC12602
LIC11755	YP_001707.1	hypothetical protein LIC11755
LIC12048	YP_001983.1	hypothetical protein LIC12048

S2 Table: Acute phase serodiagnostic antigens identified in patients with mild and severe leptospirosis.

Ag ID	Patients	Endemic controls	BHp-value	AUC	US naives	BHp-value	AUC
Mild patients							
LIC10973	1.640 (1.065/2.214)	-0.952 (-1.203/-0.701)	4.24E-03	0.765	-1.014 (-1.235/-0.793)	9.89E-05	0.872
LigB 8-12	3.757 (2.910/4.603)	0.084 (-0.014/0.182)	1.45E-04	0.809	0.287 (0.123/0.451)	8.52E-05	0.847
LigA/B 1-6[a]	3.899 (3.181/4.617)	0.537 (0.424/0.651)	1.70E-06	0.916	0.708 (0.272/1.144)	1.01E-05	0.893
LigA 8-13[a]	4.590 (3.993/5.186)	1.688 (1.192/2.183)	7.72E-05	0.851	1.413 (1.014/1.811)	1.17E-05	0.873
LIC11274	1.256 (0.864/1.649)	0.395 (0.271/0.518)	3.43E-02	0.784	0.358 (0.059/0.656)	2.75E-02	0.798
Severe patients							
LIC12544	1.438 (0.910/1.965)	-0.075 (-0.452/0.302)	1.57E-03	0.844	0.008 (-0.414/0.430)	4.13E-03	0.804
LIC10486	1.925 (1.491/2.358)	0.605 (0.317/0.893)	9.36E-04	0.851	0.691 (0.224/1.158)	9.37E-03	0.797
LIC10024	1.005 (0.792/1.218)	0.441 (0.277/0.606)	5.29E-03	0.789	0.546 (0.458/0.634)	6.04E-03	0.730
LigA/B 1-6	3.033 (2.435/3.630)	0.537 (0.424/0.651)	1.63E-06	0.935	0.708 (0.272/1.144)	3.25E-05	0.911
LigA 8-13	3.444 (2.864/4.023)	1.688 (1.192/2.183)	1.94E-03	0.811	1.413 (1.014/1.811)	1.03E-04	0.866
LIC11591	1.145 (0.992/1.297)	0.724 (0.619/0.830)	1.94E-03	0.839	0.819 (0.702/0.936)	2.38E-02	0.764
LIC12731	1.425 (1.180/1.670)	0.739 (0.473/1.004)	1.24E-02	0.831	0.691 (0.617/0.764)	1.24E-04	0.883
LIC20077	1.088 (0.731/1.445)	0.356 (0.048/0.663)	3.65E-02	0.772	0.362 (0.090/0.634)	3.19E-02	0.777
LIC20276	1.474 (1.155/1.793)	0.554 (0.414/0.694)	6.94E-04	0.901	0.777 (0.647/0.907)	5.58E-03	0.801

[a]Antigens detected in both mild and severe leptospirosis patients.

S3 Table: Convalescent phase serodiagnostic antigens identified in patients with mild and severe leptospirosis.

Ag ID	Patients	Endemic controls	BHp-value	AUC	US naives	BHp-value	AUC
Mild patients							
LIC10462	1.614 (1.195/2.034)	-0.428 (-0.743/-0.113)	1.80E-05	0.918	-0.331 (-0.535/-0.126)	2.15E-04	0.869
LigA/B 1-6 [a]	4.410 (3.741/5.079)	0.537 (0.424/0.651)	5.74E-09	0.944	0.708 (0.272/1.144)	4.86E-08	0.929
LigB 8-12 [a]	4.454 (3.779/5.130)	0.084 (-0.014/0.182)	1.83E-08	0.955	0.287 (0.123/0.451)	1.88E-08	0.946
LigA 8-13 [a]	4.973 (4.359/5.587)	1.688 (1.192/2.183)	3.11E-06	0.893	1.413 (1.014/1.811)	4.73E-07	0.902
LIC10713	1.052 (0.628/1.477)	-0.199 (-0.360/-0.038)	6.31E-04	0.865	-0.263 (-0.386/-0.140)	8.40E-04	0.849
LIC10973	1.991 (1.376/2.607)	-0.952 (-1.203/-0.701)	3.67E-04	0.795	-1.014 (-1.235/-0.793)	1.19E-05	0.896
LIC11570 [a]	3.291 (2.686/3.897)	0.565 (0.349/0.781)	1.69E-05	0.855	0.705 (0.387/1.023)	5.21E-04	0.822
LIC11694	2.725 (2.205/3.244)	0.596 (0.205/0.987)	5.95E-03	0.808	0.350 (0.095/0.604)	2.97E-03	0.792
LIC13277	1.200 (0.726/1.674)	-0.523 (-0.696/-0.350)	2.26E-03	0.809	-1.166 (-1.407/-0.924)	1.45E-06	0.909
LIC1SPN3200s2 [a]	2.352 (1.512/3.191)	0.240 (0.112/0.367)	1.50E-03	0.837	0.438 (0.227/0.649)	1.52E-02	0.779
Severe patients							
LIC10215	1.030 (0.694/1.365)	-0.663 (-0.924/-0.403)	4.17E-08	0.960	-0.712 (-0.948/-0.476)	8.30E-09	0.960
LigA/B 1-6 [a]	4.282 (3.897/4.666)	0.537 (0.424/0.651)	9.30E-17	1.000	0.708 (0.272/1.144)	3.16E-11	0.990
LigB 8-12 [a]	2.435 (1.955/2.914)	0.084 (-0.014/0.182)	4.08E-09	0.988	0.287 (0.123/0.451)	2.43E-08	0.978
LigA 8-13 [a]	5.210 (4.802/5.618)	1.688 (1.192/2.183)	7.94E-10	0.983	1.413 (1.014/1.811)	5.10E-13	0.988
LIC10486	2.761 (2.264/3.258)	0.605 (0.317/0.893)	1.29E-07	0.893	0.691 (0.224/1.158)	2.23E-05	0.883
LIC10562	2.098 (1.673/2.524)	0.540 (0.279/0.801)	6.81E-06	0.898	0.880 (0.337/1.422)	1.53E-02	0.816
LIC11222	2.164 (1.583/2.745)	0.965 (0.508/1.423)	2.39E-02	0.715	0.102 (-0.270/0.474)	1.90E-05	0.871
LIC11335	2.414 (1.790/3.037)	0.594 (0.486/0.702)	6.10E-05	0.844	0.887 (0.685/1.088)	9.70E-04	0.784
LIC11389	2.059 (1.700/2.417)	0.893 (0.552/1.234)	7.20E-04	0.836	1.173 (0.703/1.643)	4.70E-02	0.747
LIC11456	1.572 (1.214/1.929)	0.344 (0.162/0.525)	1.06E-05	0.898	0.373 (0.088/0.658)	1.67E-04	0.871
LIC11570[a]	3.319 (2.896/3.743)	0.565 (0.349/0.781)	2.70E-12	0.993	0.705 (0.387/1.023)	2.65E-10	0.980
LIC11653	1.057 (0.710/1.405)	0.190 (0.040/0.339)	8.76E-04	0.824	0.286 (0.056/0.517)	7.55E-03	0.767

LIC11893	1.645 (1.202/2.089)	0.397 (0.165/0.629)	2.64E-04	0.834	0.532 (0.301/0.763)	1.27E-03	0.779
LIC11966	2.582 (2.029/3.134)	1.204 (0.923/1.485)	1.20E-03	0.804	1.043 (0.753/1.332)	3.66E-04	0.846
LIC12180	1.113 (0.687/1.539)	0.233 (-0.125/0.591)	2.68E-02	0.730	-0.067 (-0.435/0.301)	2.54E-03	0.821
LIC12340	1.956 (1.576/2.336)	1.089 (0.768/1.409)	1.49E-02	0.749	1.180 (0.964/1.397)	1.08E-02	0.705
LIC12500	1.423 (1.201/1.645)	0.564 (0.486/0.642)	4.90E-07	0.943	0.759 (0.626/0.893)	2.07E-04	0.834
LIC12538	1.939 (1.605/2.274)	1.126 (0.820/1.432)	1.23E-02	0.752	0.981 (0.768/1.194)	4.64E-04	0.806
LIC12544	2.690 (2.168/3.212)	-0.075 (-0.452/0.302)	7.36E-09	0.958	0.008 (-0.414/0.430)	4.95E-08	0.955
LIC12631	3.609 (3.115/4.103)	1.813 (1.364/2.263)	1.09E-04	0.851	2.199 (1.734/2.663)	2.89E-03	0.797
LIC12731	1.442 (1.184/1.700)	0.739 (0.473/1.004)	7.95E-03	0.824	0.691 (0.617/0.764)	8.95E-05	0.896
LIC12921	1.982 (1.609/2.354)	0.379 (0.248/0.511)	5.26E-08	0.945	0.816 (0.579/1.052)	1.35E-04	0.839
LIC13314	1.782 (1.480/2.085)	0.909 (0.667/1.151)	1.04E-03	0.839	0.993 (0.819/1.166)	1.03E-03	0.784
LIC1SPN3200s2[a]	1.691 (1.218/2.164)	0.240 (0.112/0.367)	3.14E-05	0.938	0.438 (0.227/0.649)	4.88E-04	0.876
LIC20077	1.118 (0.757/1.480)	0.356 (0.048/0.663)	2.50E-02	0.777	0.362 (0.090/0.634)	1.80E-02	0.779
LIC20118	1.058 (0.709/1.408)	-1.233 (-1.516/-0.951)	1.28E-10	0.970	-1.438 (-1.651/-1.224)	5.10E-13	0.988
LIC20276	1.402 (1.078/1.727)	0.554 (0.414/0.694)	5.09E-04	0.888	0.777 (0.647/0.907)	1.08E-02	0.792
LIC20301	2.295 (1.854/2.737)	0.283 (0.149/0.418)	1.60E-08	0.948	0.156 (0.030/0.281)	4.29E-09	0.955

[a]Antigens detected in both mild and severe leptospirosis patients.

S4 Table: Clinical characteristics for mild leptospirosis patients with distinct antibody kinetics.

	Median (IQR) or No (%)		
	Mild patients	**Mild outliers**	**p-value**
Number	25	5	
Demographics			
Sex	11.0 (44.0)	5 (100)	0.128
Age	32.0 (18.0-44.0)	11.0 (9.0-25.5)	**0.062**
Clinical presentation			
Days of symptoms			
Acute sample collection	6.0 (3.0-8.0)	5.0 (5.0-10.5)	0.633
Convalescent sample collection	44.0 (22.0-86.5)	41.0 (27.0-71.5)	1.000
Laboratory confirmation			
Acute MAT titer	0 (0-300)	0 (0-800)	0.628
Convalescent MAT titer	200 (0-600)	3200 (400-12800)	**0.031**

5 DISCUSSÃO

Um dos maiores componentes da resposta imune adaptativa em uma infecção é a geração de imunidade humoral protetora e duradoura, mas os fatores que regem a seleção dos antígenos que são reconhecidos pelo sistema imune permanecem desconhecidos. É comum os vírus, que codificam poucas proteínas, estimularem uma resposta por anticorpos contra todas as suas proteínas estruturais. Porém, para bactérias e outros parasitas que codificam milhares de proteínas, apenas uma fração do genoma codificante é reconhecido por anticorpos do hospedeiro. No estudo de análise de enriquecimento, é descrito o uso do microarranjo de proteínas na determinação empírica do repertório de anticorpos de indivíduos naturalmente infectados por *Leptospira*. Essa técnica permite a identificação dos tipos de características estruturais, funcionais e físico-químicas que são reconhecidas mais frequentemente pelo sistema imune humano.

Os dados apresentados nesse trabalho representam uma avaliação em larga escala das proteínas de *L. interrogans* sorovar Copenhageni que são antigênicas durante a infecção natural em humanos. Para isso, foram consideradas expressas, no microarranjo, as proteínas reconhecidas pelos anticorpos anti-Histidina ou anti-Hemaglutinina, indicando a presença das caudas inseridas nas porções N-terminal e C-terminal da proteína recombinante, respectivamente. Inicialmente, o racional desta estratégia de clonagem e expressão foi identificar a expressão das proteínas na correta fase de leitura. Entretanto, foram identificadas 68 proteínas positivas apenas para cauda de hemaglutinina. Isso levanta a hipótese de que a ausência de reatividade para as caudas possa ser devida não somente à não expressão proteica, mas também à não exposição dos epítopos para o reconhecimento pelos anticorpos anti-Histidina e anti-Hemaglutinina. Assim, a detecção de apenas uma das caudas foi considerada evidência de expressão e 91% do genoma codificante predito de *L. interrogans* atendeu a este critério. Os clones referentes aos antígenos identificados como sororreativos foram posteriormente confirmados quando à integridade gênica por sequenciamento nucleotídico.

Para análise de enriquecimento, as proteínas foram classificadas como sororreativas quando (i) a média da intensidade de sinal de um grupo de amostras para uma determinada proteína foi maior do que média das reações controle

acrescida de 2,5 desvios-padrão (limite de corte) ou (ii) quando pelo menos 33% das amostras de um determinado grupo apresentaram intensidade de sinal para determinada proteína acima do limite de corte descrito acima. O racional para incluir esse segundo critério foi não descartar antígenos que fossem fortemente reconhecidos por uma pequena porção dos pacientes. Com o intuito de identificar o maior número possível de antígenos que são naturalmente reconhecidos por humanos, amostras de indivíduos saudáveis também foram submetidas aos critérios de seleção mencionados acima. Do mesmo modo, amostras longitudinais foram incluídas na tentativa de identificar antígenos com reatividade sorológica transitória.

Com isso, um total de 191 antígenos imunodominantes foram identificados, o que corresponde a 6% do genoma codificante da bactéria. Esse conjunto relativamente pequeno de proteínas representa o repertório completo de anticorpos IgM ou IgG que é gerado durante a infecção. Montar uma reposta imune contra um pequeno número de antígenos tem a vantagem de controlar a infeção de forma eficaz, com gasto energético mínimo e ainda evitar uma reação inflamatória excessiva.

Os antígenos imunodominantes foram classificados de acordo com suas características proteômicas e funcionais. A análise de enriquecimento identificou 14 características que são enriquecidas positivamente nesses antígenos sororreativos, sendo 9 comuns a ambas as classes de anticorpos IgM e IgG. As demais características foram específicas para IgM ou IgG e podem estar relacionadas à mudança de classe de IgM para IgG e à maturação de afinidade de IgG. Vale ressaltar, porém, que as características mais significativas de enriquecimento, tanto positivo quanto negativo, foram aquelas detectadas simultaneamente para IgM e IgG.

Os resultados apresentados nesse estudo estão em concordância com estudos similares previamente realizados pelo grupo com *Brucella melitensis* e *Burkholderia pseudomallei* (Felgner et al., 2009; Liang et al., 2011). As características enriquecidas positivamente com maior significância identificadas no presente estudo foram também identificadas para *B. melitensis*: predição positiva para peptídeo sinal, predição positiva para presença de 1 domínio transmembrana, COG U (secreção e tráfego intracelular) e COG N (secreção e motilidade celular). De igual forma, proteínas definidas como proteínas de superfície pelo Artemis foram enriquecidas para sororreatividade em *B. pseudomallei*. Ambas as categorias de COG N e U

definidas pelo NCBI estão relacionadas à secreção e à externalização dessas proteínas, justificando sua acessibilidade ao sistema imune. A localização sub-celular em membrana externa também torna as proteínas facilmente disponíveis aos componentes do sistema imune. De igual forma, a presença de peptídeo sinal, que é um marcador para secreção e ancoramento da proteína em membranas e a presença de 1 ou mais domínios transmembrana também foram características enriquecidas positivamente. Porém, é importante ressaltar que o algoritmo utilizado na predição de domínio transmembrana pode classificar incorretamente 20% das proteínas de bactérias Gram negativas que possuem peptídeo sinal (Krogh et al., 2001). Dessa forma, os resultados apresentados no presente trabalho podem apresentar redundância em relação a estas categorias. Proteínas citoplasmáticas foram enriquecidas negativamente para todos os três patógenos. Em conjunto, esses estudos demonstram que antígenos proteicos não são reconhecidos aleatoriamente pelo sistema imune hospedeiro; ao contrário, o sistema imune foca em tipos específicos de antígenos. A partir dessa informação e com a crescente disponibilidade de sequências genômicas completas, é possível fazer predições genéricas sobre a imunorreatividade de antígenos baseando-se apenas na sequência de aminoácidos.

Análise de sequências e a bioinformática são ferramentas que podem ser empregadas na predição de imunorreatividade, mas ambas possuem limitações que podem ser contornadas por estudos proteômicos. Por exemplo, muitos antígenos reativos não são identificados por ferramentas de bioinformática porque não se consegue classificá-los em uma das categorias normalmente empregadas nas análises de enriquecimento, o que diminui a sensibilidade da predição. Nesse estudo de análise de enriquecimento, foi observado que 25% do genoma codificante de *L. interrogans* pertence a pelo menos uma das categorias conhecidas, mas apenas 12% dessas proteínas são sororreativas. Além disso, 50% dos antígenos soro-reativos identificados não se encaixam em nenhuma das categorias existentes e, portanto, não podem ser preditos pelos métodos atuais. Dessa forma, baseado nas categorias utilizadas nesse estudo de enriquecimento, selecionando-se 25% do genoma codificante, seria possível identificar aproximadamente 50% dos antígenos imunorreativos.

Os resultados apresentados correlacionam antigenicidade com expressão *in vivo* e *in vitro* de proteínas individuais, permitindo a comparação dos dados de

microarranjo com dados de estudos prévios. Mais de 60% das proteínas antigênicas foram também detectadas por espectrometria de massas, indicando uma grande sobreposição de proteínas expressas tanto durante a infecção quando durante o cultivo *in vitro*. Entretanto, não foi observada correlação entre a concentração proteica na célula e a intensidade da sororreatividade, sugerindo que a antigenicidade de uma proteína envolve características estruturais além de abundância proteica. Essa discrepância pode também ser explicada pelas diferenças entre o nível de expressão dessas proteínas pela bactéria *in vitro* e *in vivo*, no hospedeiro humano.

Argumento similar pode ser feito para os 93 dos 108 genes diferencialmente expressos quando a bactéria é cultivada em câmaras inseridas em ratos, que não foram sororreativos no microarranjo. Por exemplo, no referido estudo, foram identificados 8 antígenos associados à virulência, dos quais apenas 4 foram sororreativos no microarranjo: LIC12631 e LIC12632 (hemolisinas), LIC10465 (LigA) e LIC11219 (peroxiredoxina). Adicionalmente, dos 18 antígenos que foram sororreativos no microarranjo e são associados à secreção (COG U) e, consequentemente, são potenciais fatores de virulência, apenas LIC10053 estava regulado positivamente no roedor reservatório. O perfil de proteínas reguladas positivamente pode diferir bastante entre o reservatório animal, que é portador crônico, e o hospedeiro humano, devido às diferentes condições ambientais e à resposta imune. A falta de correlação entre concentração proteica e antigenicidade corrobora a ideia de que genes regulados positivamente não são necessariamente antigênicos e que nem todos os fatores de virulência estimulam uma resposta imune por anticorpos.

O campo da proteômica oferece ferramentas poderosas para a identificação de antígenos sororreativos, mas também apresenta algumas limitações. A análise de enriquecimento foi baseada em ferramentas de bioinformática para classificação do genoma codificante completo de *L. interrogans* em relação a características proteicas que auxiliam na predição de antigenicidade em humanos. Os resultados apresentados são dependentes de predições computacionais e anotações genômicas. Embora diferentes versões do algoritmo de predição de peptídeo sinal utilizado tenham nos levado às mesmas conclusões, é preciso destacar que o uso de ferramentas diferentes ou mesmo possíveis erros e viés nos algoritmos empregados podem alterar significativamente os resultados descritos nesse estudo.

É possível, ainda, que o enovelamento incorreto das proteínas impressas no microarranjo interfira na reação antígeno anticorpo, tanto positiva quanto negativamente, e na correta identificação das proteínas imuno-reativas. Além disso, 9% do genoma codificante da bactéria não estava representado no microarranjo e não é possível afirmar que essas proteínas não são reativas.

Para identificação de antígenos com potencial diagnóstico e/ou vacinal e para o estudo de cinética de anticorpos, os grupos de pacientes com leptospirose sintomática foram comparados aos grupos de indivíduos saudáveis. Para tal, as proteínas foram consideradas sororreativas quando a intensidade de sinal foi duas vezes maior do que a média das reações controle (limite de corte) em mais de 60% dos indivíduos de pelo menos um dos grupos de amostras incluídos no estudo. Um total de 478 proteínas foi classificado como reativo. Com base nessas 478 proteínas, os grupos de interesse foram comparados dois a dois e as proteínas com BHp<0,05 e AUC>0,7 foram consideradas antígenos diferencialmente reativos. É importante ressaltar, porém, que devido ao uso de 2 impressoras distintas na fabricação dos microarranjos, diferentes lotes de lâminas foram produzidos e o grupo de pacientes que vieram a óbito não pôde ser comparado aos demais grupos por diferenças entre os lotes.

Quando os pacientes com leptospirose sintomática foram comparados aos indivíduos saudáveis, foram identificados 12 antígenos IgG capazes de diferenciar indivíduos com doença aguda de indivíduos não infectados residentes em região com transmissão endêmica da doença. Esses antígenos são, portanto, promissores candidatos a um novo teste laboratorial para o diagnóstico sorológico da leptospirose. Antígenos IgG específicos para pacientes foram identificados, também, em amostras de soro de fase convalescente, sendo assim potenciais candidatos a uma vacina de subunidade contra a doença.

Entre os 12 antígenos IgG com potencial diagnóstico para leptospirose aguda estão as proteínas sabidamente sororreativas LigA/B 1-6, LigA 8-13, LigB8-12 e OmpL1 (LIC10973). Inúmeros trabalhos publicados testaram as proteínas Lig como marcadores de diagnóstico para leptospirose (Matsunaga et al., 2003; Palaniappan et al., 2004; Croda et al., 2007; Silva et al., 2007; Nabity et al., 2012), assim como a OmpL1, especialmente quando em combinação com LipL21, LipL32 ou LipL41 (Sun et al., 2011). Nosso grupo identificou previamente as proteínas LIC10486 (proteína hipotética) e 12544 (proteína de ligação ao DNA) utilizando essa plataforma de

microarranjo. As 6 proteínas restantes: LIC10024 (adenilato/guanilato ciclase), LIC11591 (subunidade VII da exodesoxiribonuclease), LIC20077 (polissacarídeo desacetilase) e as proteínas hipotéticas LIC11274, LIC20276 e LIC12731 são novos candidatos diagnósticos promissores, especialmente a LIC20276, que aprimorou o desempenho diagnóstico para leptospirose grave em combinação com LigA/B 1-6. Estudos mais aprofundados precisam ser realizados para avaliar o desempenho diagnóstico desses antígenos em outras plataformas, como por exemplo, ELISA e testes rápidos.

O fato de pacientes apresentarem reatividade por anticorpos contra proteínas anotadas como hipotéticas, não somente na amostra de soro de fase aguda, mas também na amostra de fase convalescente, é um dado interessante, pois indica que, embora ainda não tenha sido atribuída função a tais proteínas, elas são, de fato, expressas pela bactéria e podem desempenhar um papel importante na infecção do hospedeiro.

Os resultados apresentados nesse trabalho são consistentes com resultados prévios obtidos pelo grupo, no qual a confecção de um microarranjo de proteínas compreendendo 61% do genoma codificante do mesmo patógeno identificou 24 antígenos sororreativos especificamente em pacientes com leptospirose grave (Lessa-Aquino et al., 2013). Dentre esses 24 antígenos IgG identificados anteriormente, 13 foram novamente detectados, reforçando seu potencial diagnóstico e validando a plataforma de microarranjo de proteínas para o descobrimento de antígenos. A inclusão de 39% do genoma codificante predito de *L. interrogans*, no entanto, não se mostrou vantajosa para identificação de novos antígenos sororreativos, uma vez que apenas 3 das 1489 proteínas e segmentos adicionados ao microarranjo de genoma codificante completo foram reativas. Isso indica que o algoritmo de seleção de antígenos utilizado no estudo prévio de microarranjo de ORFeoma parcial foi eficaz. De fato, 32 dos 36 antígenos identificados especificamente em pacientes no atual estudo (microarranjo completo) se encaixam em pelo menos uma das características descritas na análise de enriquecimento para reconhecimento por anticorpos (Liang and Felgner, 2012, 2015; Lessa-Aquino et al., 2015).

Pacientes com leptospirose e indivíduos saudáveis reagiram contra 12% do genoma codificante de *L. interrogans*. A maioria dos antígenos imunodominantes foram específicos para IgM, correspondendo a mais de 50% das proteínas

sororreativas. O elevado número de antígenos para IgM pode ser reflexo da interação antígeno-anticorpo típica de anticorpos IgM, caracterizada por ser ampla e de baixa afinidade (Ehrenstein and Notley, 2010; Landry, 2016). Tais características geralmente tornam os anticorpos do tipo IgM um indicador diagnóstico difícil de se trabalhar e de se empregar em testes diagnósticos confiáveis. A dificuldade em identificar antígenos IgM específicos para pacientes também pode estar baseada nessas características de IgM, uma vez que elas levam à uma baixa especificidade nos testes de diagnóstico sorológico devido ao *background* detectado nas amostras negativas. No presente estudo, antígenos com potencial diagnóstico ou vacinal foram identificados com sucesso através da comparação dos níveis de anticorpos IgG em pacientes e indivíduos saudáveis, mas estudo futuros precisam ser realizados para identificar antígenos reconhecidos IgM especificamente em pacientes.

No estudo prévio com o microarranjo parcial, foi evidenciado que indivíduos saudáveis que residem em área com transmissão endêmica de leptospirose apresentam alto *background* de IgG contra antígenos proteicos de Leptospira, possivelmente devido à constante exposição ao patógeno (Lessa-Aquino et al., 2013). Como é amplamente sabido que anticorpos constituem o principal mecanismo de proteção na infecção natural por Leptospira, a presença de altos títulos IgG nesses indivíduos sugere que esses anticorpos podem desempenhar papel importante na proteção contra o desenvolvimento da leptospirose clínica. Apesar desse alto fundo de reatividade IgG, foi possível identificar antígenos cuja reatividade estava ainda mais aumentada entre os pacientes hospitalizados, especialmente nas amostras de soro convalescente (Lessa-Aquino et al., 2013). De fato, a maior parte dos 36 antígenos identificados no estudo de microarranjo completo foram detectados na amostra de soro convalescente de pacientes com leptospirose grave. Um número bem menor de antígenos foi identificado em pacientes com leptospirose branda, sugerindo que a resposta IgG desses pacientes se assemelha mais à resposta dos indivíduos saudáveis que residem na mesma região.

Como não foi detectada diferença entre o número de dias de sintomas entre os pacientes com doença branda e grave no momento da coleta da amostra de soro, a diferença no perfil de anticorpos associado a cada grupo de pacientes foi devido a outros fatores que não a cinética de infecção. Baseado nisso, é possível levantar a

hipótese de que pacientes com leptospirose branda apresentam *background* de IgG que os protege contra o desenvolvimento da forma grave da doença, enquanto a ausência de tais anticorpos pode favorecer o desenvolvimento de desfechos graves nos pacientes hospitalizados. Em geral, o primeiro contato de um agente infeccioso é caracterizado sorologicamente por um aumento gradual de IgM, com pico entre os dias 7 e 10 após exposição ao patógeno, seguido de um aumento de IgG entre os dias 10 a 14. Em uma infecção secundária, porém, uma resposta robusta de IgG é rapidamente formada como consequência da ativação de células B de memória geradas durante a infecção primária (Innis et al., 1989; Hu et al., 2011; Pichichero et al., 2012). Nesse contexto, o fato de pacientes com leptospirose branda terem mantido os títulos de IgG na amostra aguda, coletada aproximadamente 5 dias após o aparecimento dos sintomas, e na amostra convalescente, coletada pelo menos 13 dias após a amostra aguda, sugere que esses pacientes montaram uma resposta anamnéstica devido a infecção secundária por Leptospira. Em contrapartida, pacientes com a forma grave da doença apresentaram uma resposta típica de uma infecção primária, com aumento nos títulos de IgG da fase aguda para fase convalescente.

Os resultados apresentados indicam que a presença de anticorpos contra proteínas de Leptospira pode conferir proteção contra leptospirose grave e que pacientes com doença branda podem ter tido infecção prévia. No entanto, inúmeros aspectos podem afetar a resposta imune do hospedeiro contra um agente infeccioso, incluindo a carga infecciosa. Pacientes com desfechos clínicos graves podem ter sido infectados por uma carga bacteriana maior que os pacientes que apresentaram a forma branda, desenvolvendo, assim, uma resposta imune mais intensa. Além disso, não é possível afirmar que os pacientes estudados nunca tenham sido infectados por *Leptospira* anteriormente, já que são provenientes de uma comunidade onde a transmissão de leptospirose é endêmica. Além disso, o painel sorológico empregado no estudo foi limitado a pacientes provenientes da cidade de Salvador, BA, Brasil. Seria interessante avaliar a resposta imune gerada por pacientes provenientes de outras regiões, infectados por diferentes espécies e/ou sorovares. Todavia, há uma necessidade de estudos deste tipo para ajudar a elucidar a resposta imune associada à infecção natural por *Leptospira* e o presente trabalho traz importantes informações para esse campo.

6 CONCLUSÕES

- Os resultados apontam que um conjunto relativamente pequeno de proteínas compõe o repertório antigênico dos anticorpos IgM ou IgG gerados durante a infecção por Leptospira em humanos;
- Os antígenos proteicos não são reconhecidos aleatoriamente pelo sistema imune hospedeiro, de modo que determinadas características aumentam a probabilidade de reconhecimento da proteína por anticorpos no hospedeiro;
- Entre as características de enriquecimento relacionadas ao reconhecimento antígeno-anticorpo, estão: predição positiva para presença de peptídeo sinal ou um domínio transmembrana, predição de localização subcelular como extracelular ou membrana externa, evidência de expressão por espectrometria de massas, regulação positiva do RNAm *in vivo* e anotação genômica relacionada ao tráfego intracelular, secreção, destinação proteica, motilidade celular, divisão celular, mecanismos de defesa, processos celulares ou envelope celular;
- Foram identificados 12 antígenos para IgG capazes de diferenciar indivíduos com doença aguda de indivíduos não infectados residentes em região com transmissão endêmica da doença, sendo, portanto, promissores candidatos a um novo teste diagnóstico para leptospirose;
- LigA/B 1-6 apresentou melhor acurácia diagnóstica, tanto em pacientes com leptospirose branda quanto em pacientes com a forma grave. A combinação com LigA 8-13 melhorou o desempenho diagnóstico para pacientes brandos, enquanto a combinação com LIC20276 foi melhor para o diagnóstico de pacientes graves;
- Foram identificados 33 antígenos cujos níveis de IgG estavam aumentados nos pacientes que se recuperaram da doença em relação aos indivíduos saudáveis. Esses antígenos são candidatos ao desenvolvimento de uma vacina de subunidade contra leptospirose;
- A maior parte dos antígenos diferencialmente reativos identificados foi detectado em pacientes com doença grave, sugerindo que pacientes com manifestações brandas apresentam uma resposta imune mais

semelhante aos indivíduos saudáveis que residem em área endêmica e reforçando o papel protetor dos anticorpos na leptospirose;

- Pacientes com diferentes formas clínicas da doença apresentaram perfis de anticorpos distintos, com variações na cinética de IgM e IgG ao longo do tempo. Em pacientes com doença grave, houve um aumento nos níveis de IgG com a progressão da doença, enquanto nos pacientes com leptospirose branda, os níveis de IgG permaneceram os mesmos;
- O perfil de cinética observado sugere que pacientes com a forma grave da doença estariam tendo uma infecção primária, caracterizada pelo primeiro contato com a leptospira, enquanto pacientes com doença branda estariam passando por infecção secundária, estando, assim, protegidos de manifestações graves pela presença de anticorpos pré-existentes.

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8 ANEXOS

Anexo I

Resumo dos antígenos identificados neste trabalho.

Antígeno	Produto	Classificação	
		Leptospirose branda	Leptospirose grave
LIC10024	adenilato/guanilato ciclase	NR	candidato diagnóstico
LIC10215	proteína hipotética	NR	candidato vacinal
LIC10462	proteína hipotética	candidato vacinal	NR
LIC10486	proteína hipotética	NR	candidato diagnóstico e vacinal
LIC10562	lipoproteína	NR	candidato vacinal
LIC10713	lipoproteína	candidato vacinal	NR
LIC10973	proteína de membrana externa	candidato diagnóstico e vacinal	NR
LIC11222	proteína hipotética	NR	candidato vacinal
LIC11274	proteína hipotética	candidato diagnóstico	NR
LIC11335	chaperonina do tipo GroEL	NR	candidato vacinal
LIC11389	proteína flagelar B	NR	candidato vacinal
LIC11456	LipL31	NR	candidato vacinal
LIC11570	proteína D de via secretora	candidato vacinal	candidato vacinal
LIC11591	subunidade VII da exodesoxirribonuclease	NR	candidato diagnóstico
LIC11694	TonB-dependent outer membrane receptor	candidato vacinal	NR

LIC12544	proteína ligadora de DNA	NR	candidato diagnóstico
LIC12731	proteína hipotética	NR	candidato diagnóstico
LIC13277	proteína hipotética	candidato vacinal	NR
LIC1SPN3200s2	NA	candidato vacinal	NR
LIC20077	polisaccarídeo desacetilase	NR	candidato diagnóstico
LIC20276	proteína hipotética	NR	candidato diagnóstico
LigA 8-13	LigA	candidato diagnóstico e vacinal	candidato diagnóstico e vacinal
LigA/B 1-6	LigA/B	candidato diagnóstico e vacinal	candidato diagnóstico e vacinal
LigB 8-12	LigB	candidato diagnóstico e vacinal	candidato vacinal

NA: anotação indisponível no NCBI

NR: não reativo

Anexo II

Protocolos de aprovação do Comitê de Ética em Pesquisa.

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisas Gonçalo Moniz

PARECER Nº 76/2005

Protocolo: 175

Projeto de Pesquisa: Um estudo de Leptospirose em Salvador, Bahia: apresentação clínica e diagnóstico microbiológico

Pesquisador Responsável: Dr. Mitermayer Galvão dos Reis

Instituição ou Departamento: LPBM/FIOCRUZ

Considerações:

Após análise ética do projeto e realização dos esclarecimentos solicitados pelo responsável, o CEP considera que o projeto atende aos princípios éticos de autonomia, beneficiência, não maleficiência, equidade e justiça.

Diante do exposto, o Comitê de Ética em Pesquisas do Centro de Pesquisas Gonçalo Moniz da Fundação Oswaldo Cruz (CEP-CPqGM/FIOCRUZ), conforme atribuições conferidas pela CONEP/CNS/MS (Carta Doc.32/04/97), com base na Resolução 196/96, julga **aprovado** o projeto supracitado.

Acrescentamos que conforme a Resolução 196/96, item VIII.4c, relativo a protocolos que envolvem áreas temáticas especiais tais como pesquisas coordenadas do exterior ou com participação estrangeira, o referido projeto será encaminhado a CONEP para análise e emissão de parecer final.

Salvador, 21 de dezembro de 2005

Dra. Marilda de Souza Gonçalves
Coordenadora
CEP – CPqGM/FIOCRUZ

TRADUÇÃO PARA O PORTUGUÊS

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3 de Fevereiro de 2009

Albert Ko, M.D.
Professor Associado de Medicina

Tipo de Submissão:	Emenda
Número do Protocolo:	9706001245
Título do Protocolo:	Leptospirose Urbana Epidêmica em Salvador, Brasil: Um estudo da apresentação clínica e desenvolvimento de métodos de diagnóstico rápido
Natureza da Emenda:	Submissão de aplicação para financiamento (NIAID/NIH 5 R43 AI072856)

Prezado Dr. Ko:

O Comitê de Revisão Institucional (IRB) realizou uma revisão diligente e aprovou a emenda do protocolo mencionado acima.

Por favor, não hesite em contatar a equipe do escritório do IRB se você tiver quaisquer questões ou necessitar de assistência para cumprir os termos desta aprovação.

Atenciosamente,

Rosemary Kraemer, Ph.D.
Diretora, Programas de Proteção de Pesquisas em Humanos

Por favor, note as importantes informações sobre esta aprovação:

- Conformidade das faturas: Esta aprovação é dependente de aderência contínua com as políticas institucionais de conformidade com as faturas.
- Informação sobre o WCMC-NYP IRBs: Os Comitês de Revisão de Institucional do Weil Cornell Medical College (WCMC)-New York Presbyterian (NYP) são constituídos conforme requisição Escritório Federal para Proteções de Pesquisas em Humanos (OHRP). O WCMC tem um Compromisso de Extensão Federal (FWA) com o OHRP. O número de FWA é FWA00000093. Ambos os IRBs da WCMC-NYP estão registrados sob esta FWA. Os números de registros para os IRBs são: IRB #1 IRB00000952; e IRB #2 IRB00004327. Se você precisar de qualquer informação adicional sobre os termos do FWA do WCMC ou dos IRBs do WCMC, por favor contate irb@med.cornell.edu.